This is a digital copy of a book that was preserved for generations on library shelves before it was carefully scanned by Google as part of a project to make the world's books discoverable online.

It has survived long enough for the copyright to expire and the book to enter the public domain. A public domain book is one that was never subject to copyright or whose legal copyright term has expired. Whether a book is in the public domain may vary country to country. Public domain books are our gateways to the past, representing a wealth of history, culture and knowledge that's often difficult to discover.

Marks, notations and other marginalia present in the original volume will appear in this file - a reminder of this book's long journey from the publisher to a library and finally to you.

Usage guidelines

Google is proud to partner with libraries to digitize public domain materials and make them widely accessible. Public domain books belong to the public and we are merely their custodians. Nevertheless, this work is expensive, so in order to keep providing this resource, we have taken steps to prevent abuse by commercial parties, including placing technical restrictions on automated querying.

We also ask that you:

+ *Make non-commercial use of the files* We designed Google Book Search for use by individuals, and we request that you use these files for personal, non-commercial purposes.

+ *Refrain from automated querying* Do not send automated queries of any sort to Google's system: If you are conducting research on machine translation, optical character recognition or other areas where access to a large amount of text is helpful, please contact us. We encourage the use of public domain materials for these purposes and may be able to help.

+ *Maintain attribution* The Google "watermark" you see on each file is essential for informing people about this project and helping them find additional materials through Google Book Search. Please do not remove it.

+ *Keep it legal* Whatever your use, remember that you are responsible for ensuring that what you are doing is legal. Do not assume that just because we believe a book is in the public domain for users in the United States, that the work is also in the public domain for users in other countries. Whether a book is still in copyright varies from country to country, and we can't offer guidance on whether any specific use of any specific book is allowed. Please do not assume that a book's appearance in Google Book Search means it can be used in any manner anywhere in the world. Copyright infringement liability can be quite severe.

About Google Book Search

Google's mission is to organize the world's information and to make it universally accessible and useful. Google Book Search helps readers discover the world's books while helping authors and publishers reach new audiences. You can search through the full text of this book on the web at `http://books.google.com/`

LIBRARY
OF THE
UNIVERSITY
OF
CALIFORNIA

Chemistry Lib.

BOUGHT FROM

CHEMISTRY LIBRARY

N S'

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

OFFICIAL ORGAN OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

EDITED BY

ANLEY R. BENEDICT, New York, N. Y.
LAFAYETTE B. MENDEL, New Haven, Conn.
RY D. DAKIN, Scarborough, N. Y.
DONALD D. VAN SLYKE, New York, N. Y.

WITH THE COOPERATION OF THE EDITORIAL COMMITTEE

OTTO FOLIN, Boston, Mass.
WALTER JONES, Baltimore, Md.
L. J. HENDERSON, Cambridge, Mass.
GRAHAM LUSK, New York, N. Y.
ANDREW HUNTER, Toronto, Canada
THOMAS B. OSBORNE, New Haven, Conn.
WALTER W. PALMER, Baltimore, Md.
A. N. RICHARDS, Philadelphia, Pa.
L. L. VAN SLYKE, Geneva, N. Y.

VOLUME XLIV
BALTIMORE
1920

COPYRIGHT 1920
BY
THE JOURNAL OF BIOLOGICAL CHEMISTRY

Chemistry Lib.

PUBLISHED BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH FOR THE
JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

QP501
J72
v. 44
CHEMISTRY
LIBRARY

CONTENTS OF VOLUME XLIV.

No. 1. October, 1920.

Osborne, Thomas B., and **Mendel**, Lafayette B. Skimmed milk as a supplement to corn in feeding.......... 1
Bailey, C. H., and **Gurjar**, A. M. Respiration of cereal plants and grains. II. Respiration of sprouted wheat.......... 5
Bailey, C. H., and **Gurjar**, A. M. Respiration of cereal plants and grains. III. Respiration of rice paddy and milled rice.......... 9
Bailey, C. H., and **Gurjar**, A. M. Respiration of cereal plants and grains. IV. The respiration of frosted wheat plants.......... 13
Bailey, C. H., and **Gurjar**, A. M. Respiration of cereal plants and grains. V. Note on the respiration of wheat plants infected with stem rust.......... 17
Gore, H. C. Occurrence of diastase in the sweet potato in relation to the preparation of sweet potato syrup.......... 19
Sherman, H. C. Calcium requirement of maintenance in man.......... 21
Peterson, W. H., and **Fred**, E. B. The production of acetaldehyde by certain pentose-fermenting bacteria.......... 29
Myers, Victor C., and **Short**, James J. The estimation of chlorides in blood.......... 47
Bell, Richard D., and **Doisy**, Edward A. Rapid colorimetric methods for the determination of phosphorus in urine and blood.......... 55 ✓
Underhill, Frank P., and **Simpson**, George Eric. The effect of diet on the excretion of indican and the phenols.......... 69
Rose, William C., and **Bodansky**, Meyer. Biochemical studies on marine organisms. I. The occurrence of copper.......... 99
Souza, Geraldo de Paula, and **McCollum**, E. V. A study of the factors which interfere with the use of yeast as a test organism for the antineuritic substance.......... 113
Haggard, Howard W. Hemato-respiratory functions. VI. The alteration of the CO_2 ratio (H_2CO_3:$NaHCO_3$) in the blood during elevation of body temperature.......... 131
Mattill, H. A., and **Conklin**, Ruth E. The nutritive properties of milk, with special reference to reproduction in the albino rat.......... 137
Miller, Elizabeth W. The effect of cooking on the water-soluble vitamine in carrots and navy beans.......... 159
Whipple, Bertha K. Water-soluble B in cabbage and onion.......... 175
Hart, E. B., and **Humphrey**, G. C. Can "home grown rations" supply proteins of adequate quality and quantity for high milk production? II.......... 189

M589513

Denis, W., and **Aldrich, Martha.** Note on the preservation of specimens of blood intended for blood sugar determinations.... 203

No. 2. November, 1920.

Mann, Hubert. An apparatus for continuous dialysis or extraction. 207
Prentice, W., **Lund, H. O.**, and **Harbo, H. G.** An attempt toward the determination of the total alkaline reserve of the body.... 211
Kipp, Harold A. Variation in the cholesterol content of the serum in pneumonia.... 215
Cameron, A. T., and **Hollenberg, M. S.** The nature of chlorine combination in urine.... 239
Imrie, C. G., and **Graham, S. G.** The fat content of embryonic livers. 243
Karr, Walter G. Some effects of water-soluble vitamine upon nutrition.... 255
Karr, Walter G. Metabolism studies with diets deficient in water-soluble (B) vitamine.... 277
Johns, Carl O., and **Jones, D. Breese.** Some amino-acids from the globulin of the coconut as determined by the butyl alcohol extraction method of Dakin.... 283
Jones, D. Breese, and **Johns, Carl O.** Hydrolysis of the globulin of the coconut, *Cocos nucifera*.... 291
Johns, Carl O., and **Waterman, Henry C.** Some proteins from the mung bean, *Phaseolus aureus* Roxburgh.... 303
Lynch, Vernon. Chemistry of the whitefish sperm.... 319
Collip, J. B. The alkali reserve of marine fish and invertebrates. The excretion of carbon dioxide.... 329
Horiuchi, Yajiro. Studies on blood fat. I. Variation of the blood fat constituents of rabbits under normal conditions.... 345
Horiuchi, Yajiro. Studies on blood fat. II. Lipemia in acute anemia.... 363
Daniels, Amy L., and **Loughlin, Rosemary.** A deficiency in heat-treated milks.... 381
Bodansky, Meyer. Biochemical studies on marine organisms. II. The occurrence of zinc.... 399
Tisdall, Frederick F. Estimation of the phenolic substances in urine.... 409
Anderson, R. J. Composition of inosite phosphoric acid of plants. Seventeenth paper on phytin.... 429
Robertson, T. Brailsford, and **Ray, L. A.** Experimental studies on growth. XVI. The influence of brain tissue, freed from cholesterol, upon the growth of the white mouse.... 439
Lewis, D. Sclater, and **Mason, Edward H.** The diastatic ferments of the blood.... 455
Arzberger, C. F., **Peterson, W. H.**, and **Fred, E. B.** Certain factors that influence acetone production by *Bacillus acetoaethylicum*.... 465

Miller, Emerson R. Dihydroxyphenylalanine, a constituent of the velvet bean.... 481
Funk, Casimir, and Dubin, Harry E. A test for antiberi-beri vitamine and its practical application. Plate 1.... 487
Dakin, H. D. Amino-acids of gelatin.... 499
Swoboda, Frederick K. A quantitative method for the determination of vitamine in connection with determinations of vitamine in glandular and other tissues.... 531
Bradley, H. C., and Felsher, H. Studies of autolysis. VI. Effect of certain colloids upon autolysis.... 553
Wishart, Mary B. Experiments on carbohydrate metabolism and diabetes. III. The permeability of blood corpuscles to sugar. 563
Parsons, Helen T. The antiscorbutic content of certain body tissues of the rat. The persistence of the antiscorbutic substance in the liver of the rat after long intervals on a scorbutic diet.. 587
McCollum, E. V., and Parsons, Helen T. The antiscorbutic requirement of the prairie dog. Plates 2 and 3.... 603
Index to Volume XLIV.... 609

SKIMMED MILK AS A SUPPLEMENT TO CORN IN FEEDING.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Alfred J. Wakeman.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, July 30, 1920.)

Practical experience has shown that corn-meal and skimmed milk form a combination which promotes the rapid growth of young animals as well as poultry. Laboratory experience has lately shown that the good results obtained with this mixture are due to the fact that the nutritive deficiences of the corn-meal are made good by the constituents of the milk. As the high cost of these food products makes it important to use them in the most efficient proportions, we undertook the experiments described in this paper. These were made with the following considerations in mind.

The food intake of animals is chiefly determined by their calorific requirements. In agricultural practice corn or corn-meal usually furnishes the major part of the calories of the ration. If skimmed milk is fed separately and before the corn-meal the animal may readily eat more than enough to supplement properly the corn, if fed milk after the corn it may eat too little. The proper way to feed these products, therefore, would be to mix them so that both may be eaten at the same time. Such a method would be particularly advantageous for those who have an irregular supply of skimmed milk because in this way all the latter would be used under conditions of maximum efficiency. Furthermore each individual animal would get more nearly its proper quota of the

---

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

milk than if it were allowed to drink it from a trough in company with others.

Our first experiment was designed to show how efficient a mixture of equal weights of skimmed milk and corn-meal would be and what deficiencies, if any, it might have. We intended to repeat this experiment using a smaller proportion of skimmed milk if the mixture of equal parts proved adequate, but this has been rendered unnecessary by experiments recently described by Hart and Steenbock[1] who state that

> " . . . . a highly efficient protein mixture is not obtained until the proportion of liquid milk to corn-meal reaches 1:1; in this proportion the milk nitrogen will constitute approximately 30 per cent of the total nitrogen of the ration.
>
> These data relate only to protein efficiency and of course are not to be interpreted as indicating the amount of milk necessary for providing an adequate supply of vitamines or salts to an animal with a growth rate similar to that of swine."

Since their experiments supplement ours and ours supplement theirs in respect to the features indicated we here present our results with the approval of Hart and Steenbock.[2]

We fed four albino rats on a mixture of 91.5 per cent of yellow corn-meal and 8.5 per cent of "Krystalak," a preparation of dried skimmed milk containing 35 per cent of protein (N × 6.38) and 2 per cent of fat. The skimmed milk thus furnished one-quarter of the 11.55 per cent of protein which the food contained, while the milk fat was equal to only 0.16 per cent of the food mixture. The Krystalak contained 8.37 per cent of ash, thus contributing to the food mineral salts equal to only 0.7 per cent of the mixture.

Hart and Steenbock's experiments show that the protein in this food should be adequate, both in quality and quantity. According to Steenbock and Boutwell,[3] 88 per cent of yellow corn-meal supplies enough of the fat-soluble vitamine for normal growth and reproduction of albino rats when a salt mixture containing sodium chloride, calcium lactate, and ferric citrate is also added, and the corn proteins are supplemented with casein. We should therefore expect our food mixture to be adequate in respect to this food accessory. Since 71 per cent, or more, of cornmeal has been shown by McCollum, Simmonds, and Pitz[4] to sup-

---

[1] Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, 1920, xlii, 167.

[2] As these authors have stated that they have complete experiments with rats similar to those they have reported with pigs, we did not feel at liberty to anticipate their publication without consulting them.

[3] Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 81.

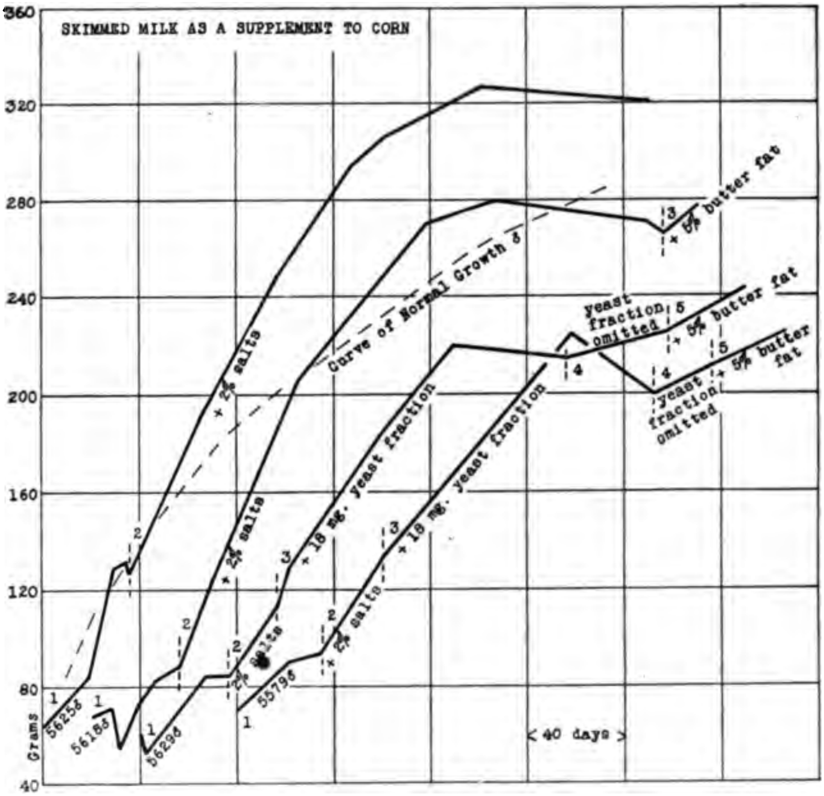


CHART 1. Showing the effect of supplementing corn with milk in the food of growing rats. In Period 1 growth was limited by the lack of sufficient suitable salts in the mixture. When these were supplied in Period 2 and subsequently by addition of 2 per cent of our salt mixture, good growth was secured. Addition of a vitamine preparation obtained from yeast (Rats 5629, 5579) did not exhibit any advantage, thus suggesting that skimmed milk and corn in the proportions used furnish sufficient water-soluble vitamine. Other details are referred to in the text.

---

[4] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxviii, 153.

ply enough of the water-soluble vitamine for the normal growth of albino rats, there is no reason to suspect that our food mixture is deficient in this respect.

The only question that deserves serious consideration is whether or not the salts supplied by the milk sufficed to supplement adequately the known mineral deficiencies of the corn. In the proportions used the 8 per cent of Krystalak contributed only 0.7 per cent of inorganic matter to the ration. The results of our experiments show that this is not enough, as it was necessary to add some of our standard salt mixture[5] before normal growth was made.

Chart 1 shows that Rat 5625 grew at a rate greater than the normal without any other addition to the diet than the salt mixture. Under the same conditions Rat 5618 grew somewhat better than at the normal rate for 160 days, but during the next 80 days it lost a little weight. This was recovered within 2 weeks when 5 per cent of butter fat was added to its ration. During a considerable part of the time Rats 5629 and 5579 received 18 mg. daily of a concentrated preparation of the water-soluble vitamine from yeast, an amount previously proved to supply alone enough of this vitamine for normal growth. The chart shows no beneficial effect after the addition of this preparation, or any unfavorable effect when it was discontinued. These rats did not grow so well as the other two, their rapid early gains during the first 130 days being followed by very slow growth. After 218 days of feeding, the addition of 5 per cent of butter fat caused no better growth.

These experiments indicate that this mixture of yellow corn-meal, skimmed milk solids, and salts may be slightly deficient in the fat-soluble vitamine. In practice such a mixture rarely forms the sole diet of young animals and when, as is generally the case, some green fodder is eaten this possible deficiency would be provided for. It is possible that the deficiency in mineral matter would also be compensated in this way, but it would be well to add about 1 pound each of calcium carbonate and sodium chloride[6] per 100 pounds of corn-meal to be on the safe side.

---

[5] The composition of this salt mixture is given by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

[6] *Cf.* McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 165.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

II. RESPIRATION OF SPROUTED WHEAT.

By C. H. BAILEY and A. M. GURJAR.

(*From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, St. Paul.*)

(Received for publication, June 21, 1920.)

Respiration in frost-damaged wheat has been observed by us[1] to be at a higher level than in normal or sound wheat of the same moisture content. This was believed to be due, in part at least, to the higher percentage of dextrose and other simple carbohydrates in the frozen wheat. It then appeared desirable to determine the effect of sprouting upon the respiration of the grain, to ascertain whether or not this form of unsoundness in bulk grain could be expected to affect its keeping qualities.

A quantity of normal hard spring wheat was divided into three portions: one of which was wetted and allowed to sprout for 24 hours in the room at a temperature of ±20°; the second was sprouted under the same conditions for 48 hours; while the third was used in its original state as a control. The sprouted wheats were rapidly dried by spreading them out in a thin layer and blowing air over and through them with an electric fan. They were thus reduced to a moisture content of about 12 per cent. Each of the three samples was divided, and the several portions brought to different percentages of moisture by the addition of varying quantities of water. After standing for 3 days each of these was sealed in a tower, incubated at 37.8°C. for 4 days, and the respired CO_2 determined.

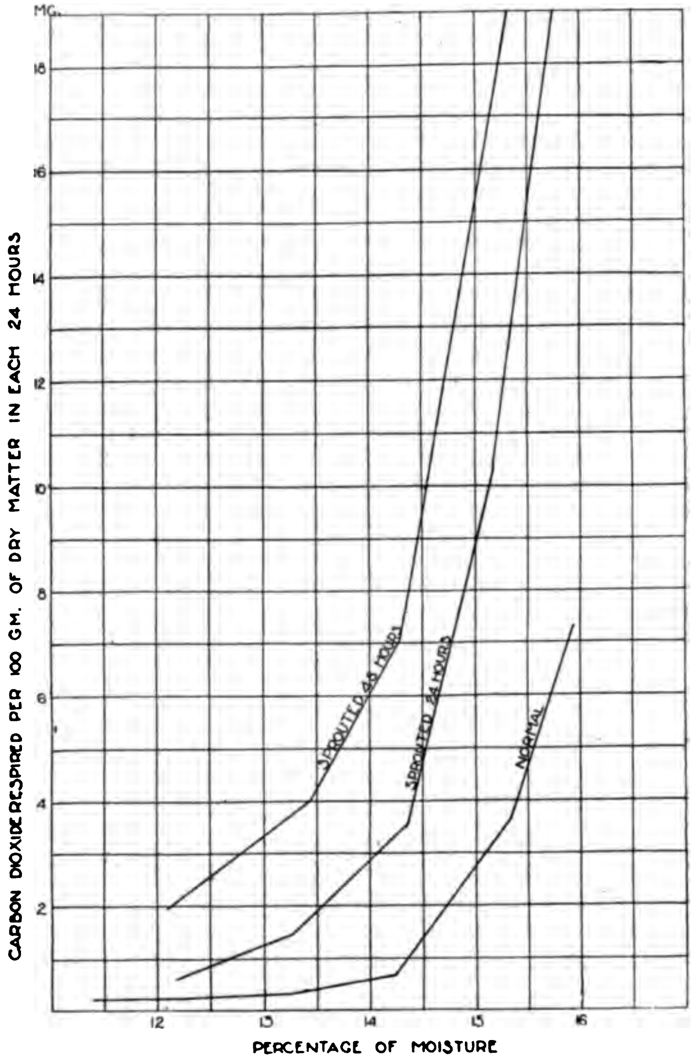
Chemical analysis of the several samples showed the normal wheat to contain 0.16 per cent of reducing sugars (calculated as

---

* Published with the approval of the Director as Paper 204, Journal Series, Minnesota Agricultural Experiment Station.

[1] Bailey, C. H., and Gurjar, A. M., Respiration of stored wheat, *J. Agric. Research*, 1918, xii, 685 (note pp. 697–699).

ctrose), while the samples germinated 24 and 48 hours con-
ned 0.59 per cent and 1.11 per cent respectively, the ratios
the three samples being 14.4 : 53.2 : 100 in the order named.



ı. **1. Graph showing the relative rates of respiration of normal and sprouted wheat.**

The quantity of CO_2 respired by each of these samples with different percentages of moisture is shown in Table I and in Fig 1. The latter indicates that at moisture contents of 12 to 16 per cent the sprouted grain respires decidedly more vigorously than the normal grain. At 12.5 to 14 per cent of moisture the ratios are approximately 1 : 4 : 10 for the normal, the 24 hour, and the 48 hour sprouted samples, which are not far different from the ratio of their reducing sugar content.

TABLE I.

Respiration of Normal and Sprouted Spring Wheat.

Normal.		Sprouted 24 hrs.		Sprouted 48 hrs.	
Moisture.	CO_2 respired per 100 gm. of dry matter in each 24 hrs.	Moisture.	CO_2 respired per 100 gm. of dry matter in each 24 hrs.	Moisture.	CO_2 respired per 100 gm. of dry matter in each 24 hrs.
per cent	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*
11.39	0.216	12.19	0.615	12.10	1.950
12.27	0.255	13.26	1.454	13.44	4.010
13.24	0.320	14.36	3.580	14.28	7.032
14.23	0.680	15.16	10.310	15.87	24.720
15.33	3.660	16.01	24.270		
15.94	7.320				

The shapes of the curves are somewhat different as well. Thus we find the acceleration of respiration with increasing moisture to be rather gradual between 12 and 14.5 per cent of moisture in the case of normal wheat, while the sprouted wheats show much greater acceleration between these limits.

These data establish that respiration, and consequently the quantity of heat energy released per unit of time and material, proceeds at a higher rate in sprouted grain than in normal wheat. This would be of significance in commercial storage of bulk cereals, and in grading grain when the grading system takes cognizance of respiration and keeping qualities in storage.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

III. RESPIRATION OF RICE PADDY AND MILLED RICE.

By C. H. BAILEY and A. M. GURJAR.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, St. Paul.)

(Received for publication, June 21, 1920.)

In our paper on the respiration of stored wheat[1] we discussed the seat of respiration in the wheat kernel. It appeared, from published data, that respiration was most active in the germ or embryo, and lowest in the endosperm. To test this hypothesis further, we secured a series of samples representing rice paddy, and its milled products at different stages of milling. These were furnished by Mr. J. Mitchell Jenkins, Superintendent of the Rice Experiment Station at Crowley, Louisiana, who kindly obtained them from one of the Crowley rice mills. The rice was of the Blue Rose variety, 1,000 average kernels of which weighed 29.67 gm.

We proceeded to compare the respiration of the rough rice or paddy with that of the rice from the "stone" and from the "huller." That from the stone had practically all the hulls removed, as well as part of the germ, while the rice from the huller had advanced another stage in the milling process, and had most of the bran and germ removed. Portions of each of these three kinds of material were brought to different percentages of moisture by the addition of varying quantities of water. The treatment accorded them and the method of determining respiratory activity were similar to those employed in the wheat studies reported in the paper mentioned above.

In Tables I and II and Fig. 1 is shown the relative respiratory activity of these samples. Fig. 1 and Table II indicate plainly that the rice paddy respires much more vigorously than the milled rice, and that, as the milling proceeds, the rate of respiration

---

* Published with the approval of the Director as Paper 205, Journal Series, Minnesota Agricultural Experiment Station.

[1] Bailey, C. H., and Gurjar, A. M., Respiration of stored wheat, *J. Agric. Research*, 1918, xii, 685.

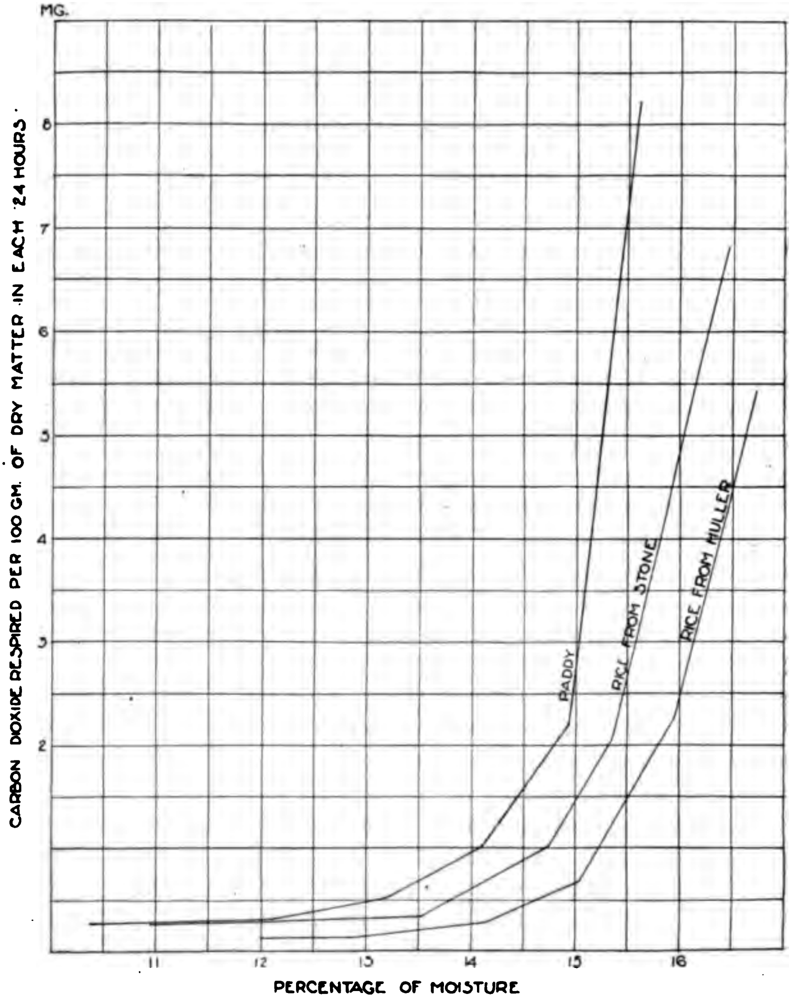


Fig. 1. Graph showing the relative rates of respiration of rice paddy, rice from stone, and rice from huller.

diminishes. Thus, the rice from the stones respired approximately 0.6 and that from the huller about 0.3 times as vigorously as the paddy.

Since the data presented in Table I were of necessity obtained in working with lots which did not vary uniformly in moisture content, the respiratory activity of each lot at even percentages of moisture was computed. These interpolated data are given in Table II, and establish the relative respiratory activity of the several lots more satisfactorily than the values given in Table I.

TABLE I.

Respiration of Paddy and Milled Rice Incubated at 37.8°C. for 4 Days.

Paddy.		Rice from stone.		Rice from huller.	
Moisture.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.	Moisture.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.	Moisture.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.
per cent	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*
10.37	0.27	10.93	0.27	11.99	0.13
12.15	0.32	13.54	0.35	12.87	0.14
13.22	0.52	14.75	1.03	14.14	0.29
14.12	1.01	15.35	2.06	15.03	0.68
14.93	2.20	16.47	6.82	15.95	2.27
16.11	8.22			16.73	5.43

TABLE II.

Interpolated Quantity of Carbon Dioxide Respired per Unit of Time and Material at Even Percentages of Moisture.

Sample.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.				
	12 per cent moisture.	13 per cent moisture.	14 per cent moisture.	15 per cent moisture.	16 per cent moisture.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Paddy..............	0.32	0.48	0.95	2.56	7.65
From stones........	0.29	0.32	0.60	1.46	4.70
" huller........	0.13	0.15	0.27	0.67	2.44

The acceleration of respiration with increase in moisture content was then computed, using, as in the studies on wheat, the formula $\frac{K_m - K_{m-1}}{K_{m-1}}$ in which K_m represents the rate of respiration at a particular percentage of moisture, and K_{m-1} represents the rate of respiration of the same sample containing 1 per cent of moisture less than K_m. These data are given in Table III and show that the acceleration due to increasing moisture is much the same in all three samples. This is also indicated by Fig. 1. The acceleration, within the limits studied, is materially greater than was found in our work on wheat.[2]

TABLE III.

Acceleration of Rate of Respiration of Rice Paddy and Milled Rice with Increasing Moisture $\frac{K_m - K_{m-1}}{K_{m-1}}$.

Sample.	Acceleration between the following percentages of moisture.			
	12 to 13 per cent.	13 to 14 per cent.	14 to 15 per cent.	15 to 16 per cent.
Paddy................	0.50	0.97	1.69	1.99
From stones..........	0.10	0.88	1.43	2.22
" huller...........	0.15	0.80	1.48	2.64

The results of this study appear to support our previous conclusion that the germ or embryo is the seat of the most active respiration in a typical grain. It also shows that with different cereals the relative acceleration of respiration with an increase in the moisture content of the grain varies with different species, being greater in rice and its milled products than in hard wheat.

---

[2] Bailey and Gurjar,[1] p. 694.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

IV. THE RESPIRATION OF FROSTED WHEAT PLANTS.

By C. H. BAILEY and A. M. GURJAR.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, St. Paul.)

(Received for publication, June 21, 1920.)

In a previous paper[1] we presented the results of a study of the respiration of wheat which had been frozen in the field before ripening. It appeared that the grain of such wheat, which had been thus frozen and subsequently thawed, respired more vigorously when stored in bulk than did normal wheat of the same moisture content. The greatest proportional difference was observed when the respective samples contained between 14.0 and 16.5 per cent of moisture.

To account for these differences between frosted and normal or sound wheat, it was postulated that, after freezing and then thawing, hydrolysis of certain complex carbohydrates probably proceeded at an increased rate. The products of this hydrolysis, and particularly the glucose thus produced, apparently constitute the substrate for the respiratory enzymes. The increased proportion of substrate may have accelerated the reaction induced by these enzymes and thus resulted in the respiration of more CO_2 per unit of time, and consequently the liberation of more heat energy.

It appeared that, if this hypothesis was sound, we should find that on freezing and then thawing the entire plant before it ripened a difference in the respiration of such material and of plants not frozen should be observed. We accordingly selected what appeared to be average plants in a field of Minnesota No.

---

* Published with the approval of the Director as Paper 206, Journal Series, Minnesota Agricultural Experiment Station.

[1] Bailey, C. H., and Gurjar, A. M., Respiration of stored wheat, *J. Agric. Research*, 1918, xii, 685.

169 bluestem wheat. Plants were cut at different stages of growth, the stems being severed 4 to 5 cm. above the ground level. This was done at 9.30 a.m. Half of these plants were immediately frozen by placing them in a refrigerating plant at a temperature of about −6°C. The following morning they were thawed in the air and then sealed in long glass tubes of about 5 cm. internal diameter. The other half of the plants, which were not frozen, were placed immediately after harvesting in similar glass tubes. Both lots were incubated in a dark thermostat for 24 hours at 30° and the respired CO_2 was determined by absorption in $Ba(OH)_2$ in the Truog tower as described by Gurjar.[3]

From the data thus secured it appeared that the frozen plants respired more vigorously during the first 24 hours after thawing than did plants which had not been frozen. The proportional difference is not so great as was observed in the stored grain. It is possible, however, that the differences would have been greater had measurements been made after the material had stood a longer time after thawing.

TABLE I.

Respiration of Normal and Frozen Wheat Plants.

Date cut.	Moisture in plant.	Weight per 1,000 kernels of dried grain.	CO_2 respired per 100 gm. of dry matter in 24 hrs.	
			Frozen plants.	Normal plants.
1917	*per cent*	*gm.*	*mg.*	*mg.*
Aug. 8	51.57	21.89	3,719	2,852
" 15	43.54	24.0	2,486	1,855
" 20	35.03	29.5	1,811	1,722

The data given in Table I include the average percentage of moisture in the plants as cut, the weight per 1,000 kernels of the grain in the head after it had been cut and dried, and the CO_2 respired by the frozen and thawed, and the unfrozen plants. The grain was nearly ripe enough to harvest at the time the samples were taken on August 20, 1917. This is more or less evident from the weight per 1,000 kernels on that date, which is as great as the average weight of a similar number of ripe kernels.

---

[3] Gurjar, A. M., The adaptation of Truog's method for the determination of carbon dioxide to plant respiration studies, *Plant World*, 1917, xx, 288.

While no sweeping conclusions can be based on these data, they suggest that the extent of damage to the grain, as viewed from the milling and baking standpoint, might be indicated by the relative difference in respiration between plants frozen and thawed, and plants harvested at the same time but not frozen. As the plants approach maturity the relative difference between plants treated in these ways materially diminishes. The damage to the grain is generally conceded to diminish similarly. When a point is reached in the maturing of the plant where freezing and thawing do not accelerate respiration, it is highly probable that the grain will not be affected in its milling qualities by such treatment. That the moisture content plays an important part is indicated by the data. Desiccation accompanying maturity suppresses the activity of the enzymes responsible for respiration, and doubtless similarly suppresses the hydrolytic enzymes which unfavorably affect the quality of the grain.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

V. NOTE ON THE RESPIRATION OF WHEAT PLANTS INFECTED WITH STEM RUST.

By C. H. BAILEY and A. M. GURJAR.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, St. Paul.)

(Received for publication, June 21, 1920.)

During the crop season of 1917, a series of studies was conducted involving the respiration of wheat plants. In one phase of this work a number of rust-infected Marquis wheat plants were selected about 2 weeks before maturity, and cut a few centimeters above the crown of the plant. These plants were severely infected, being rated at between 80 and 90 per cent on the scale employed by the office of Cereal Investigations, United States Department of Agriculture.

The harvesting was done at 9.30 a.m., and the plants were sealed at once in glass tubes. At the same time a like number of plants of the same variety not infected with rust were cut and placed in similar tubes. Duplicates of both lots were secured, and these were placed at once in a dark thermostat at 30°C. After 24 hours the tubes were removed and the respired CO_2 was determined.

In Table I are shown the moisture content of the plants, weight of 1,000 kernels after drying, and milligrams of carbon dioxide respired in 24 hours (*a*) per 100 gm. of dry matter, and (*b*) per plant. The rust-infected plants had a decidedly lower moisture content than the sound plants on the day they were cut (August 9, 1917), due, presumably, to the accelerated transpiration of rust-infected tissues.[1] The moisture content was actually as low as that of normal plants in an adjoining field a week later. The

---

* Published with the approval of the Director as Paper 207, Journal Series, Minnesota Agricultural Experiment Station.

TABLE I.

Respiration of Normal and Rust-Infected Marquis Wheat Plants Harvested August 9, 1917.

Sample.	Moisture in plant.	Weight per 1,000 kernels of dried grain.	CO_2 respired in 24 hrs.	
			Per 100 gm. of dry matter.	Per plant.
	per cent	*gm.*	*mg.*	*mg.*
Normal plants	56.08	19.54	2,904	61.46
Rust-infected plants	46.41	20.44	1,608	39.16

kernels in the heads of the infected plants were somewhat heavier, although the difference is not significant. The rate of respiration of the infected plants was materially lower than the sound plants. If respiration of these tissues is in any sense a measure of metabolic processes it appears that metabolism was decidedly depressed in the rust-infected plants. This may afford an index to the metabolism of such infected material, and the consequent effect upon the filling of the kernel.

OCCURRENCE OF DIASTASE IN THE SWEET POTATO IN RELATION TO THE PREPARATION OF SWEET POTATO SYRUP.

By H. C. GORE.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, July 18, 1920.)

The manufacture of syrup from sweet potatoes by the use of malt has long been a matter of public record.[1] Directions for the small scale production of sweet potato malt syrup were issued by the Department of Agriculture in February, 1919. The necessity for use of malt, however, apparently has prevented any wide use of the method.

Further work on the production of the syrup has unexpectedly revealed the fact that the sweet potato is so rich in diastase that nearly all the starch becomes converted into soluble carbohydrates by autolysis upon slow cooking. Moreover, the hot pulp formed by crushing the cooked sweet potatoes drains readily, thus permitting the easy recovery of the sweet juices. Sweet potato syrup, therefore, can easily be made without the use of malt.

The method consists simply in so heating the potatoes in the water that the tissues are heated at the temperature of maximum diastatic activity for from 10 to 20 minutes, then heating to boiling in order to soften the tissues, crushing, and separating the sweet juices from the insoluble pulp. The juice is then evaporated to syrup with or without further treatment.

For example, 1 kilo of Porto Rico potatoes was covered with water in an aluminum kettle, placed over a Fletcher burner, and the water heated to 60°C. The gas was then turned down and the heat applied very slowly so that the temperature gradually rose from 60 to 80°C. during an hour and from 80° to the boiling point during the next ½ hour. The boiling was continued for ½ hour, when the potatoes were thoroughly soft. They were then mashed in the water in which they were cooked and enough water was added to form a thin pulp. The sweet juice in the pulp was then freed from the insoluble pulp ingredients by suction, using a Buchner funnel and filter paper, and the residue on the filter repeatedly exhausted with hot water. The filtrates were combined, evaporated to a thin syrup, and weighed. The weight was 714 gm. and the Brix reading at 20°C. was 37.6°. Thus, 714 × 37.6 or 268.5 gm. of syrup solids had been exhausted from 1 kilo of sweet potatoes. The dried pulp weighed 59 gm. The syrup was finally evaporated to a solids content of about 75 per cent. It was a slightly turbid, amber-colored liquid with a faint, pleasant odor and a sweet taste, with a slight flavor of the sweet potato. It contained a little soluble starch as shown by the iodine test, but not enough to cause it to thicken upon standing.

---

[1] U. S. Patent 109,991 was granted to Charles Delamarre on December 6, 1870.

The sweet potatoes can be prepared for extraction by simply cooking them in water as in preparing for table use, but the resulting syrup will contain enough soluble starch to cause it to thicken upon cooling and standing. Tests on the diastatic power of extracts of sweet potato and sweet potato flour were made by Lintner's method as described by Brown.[2]

	Degrees Lintner
Porto Rico fresh pulp[3]	300
Nancy Hall " " [3]	125
Porto Rico flour[4]	500
Nancy Hall " [4]	300
Big Stem Jersey flour[4]	160

SUMMARY.

Sweet potatoes are high in diastatic power and it is possible to convert nearly all their starch into soluble carbohydrates by slowly cooking the potatoes in water. The pulp formed by mashing the cooked potatoes with hot water drains readily, permitting the easy recovery of the sweet juice.

---

[2] Brown, A. J., Laboratory studies for brewing students, New York, 1904.

[3] Reduced to pulp in a Herles press (Herles, F., *8th Internat. Congr. Appl. Chem.*, 1913, xxvi, 5).

[4] Produced by shredding sweet potatoes with beet knives, drying in a rapid current of hot air, grinding, and bolting.

CALCIUM REQUIREMENT OF MAINTENANCE IN MAN.*

By H. C. SHERMAN.

With the Cooperation of A. R. Rose and M. S. Rose.

(From the Department of Chemistry, Columbia University, New York.)

(Received for publication, July 29, 1920.)

The demonstration upon laboratory animals by Osborne and Mendel,[1] that calcium necessarily occupies a prominent place in the inorganic food supply, and that of McCollum, Simmonds, and Parsons,[2] that calcium is one of the limiting factors in a very large proportion of our staple foods, lends added interest to the measurement of the calcium requirement in man and the comparison of the amount of calcium required for normal human nutrition with the amounts found in the ordinary freely chosen food of representative families or other groups of people.

Results obtained in this laboratory several years ago[3] indicated that, in so far as could be judged from the limited data determined at that time, it was by no means safe to assume that a freely chosen food supply would always furnish calcium in quantity sufficient to afford a safe margin above the actual requirements of normal nutrition.

Further study, therefore, seemed desirable and the purpose of the present paper is to summarize the data of a considerable number of additional laboratory experiments and to compare them with the findings of further observations upon actual food supplies.

---

* Published as Contribution No. 342 from the Department of Chemistry, Columbia University.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.

[2] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 113, and in numerous other papers.

[3] Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Off. Exp. Stations, Bull. 227*, 1910.

In addition to the experimental data published from this laboratory in 1910,[3] 1918,[4] and 1919,[5] of which the latter have been recorded without as yet being discussed, studies upon the calcium requirement of maintenance have been made upon three healthy men, Subjects I, E, and R.

Subject I, weighing 61 kilos, consumed bread, milk, wheat breakfast food, egg whites, apple (or banana), rice, butter, and coffee, his daily calcium intake during each of six successive 4 day experiments being 0.46, 0.41, 0.41, 0.42, 0.32, 0.32 gm., respectively. The corresponding output in each of the six experiments was: in urine, 0.15, 0.18, 0.20, 0.20, 0.20, 0.20; in feces, 0.42, 0.50, 0.26, 0.34, 0.32, 0.21 gm. per day, respectively (Experiments 10 to 15 in Table I).

Subject E, weighing 69 kilos (Experiments 56 to 58 in Table I), after a preliminary period on calcium-poor food, took during a 3 day experiment a basal ration of bread, butter, and fruit with a calcium intake of 0.18 gm. per day; the daily output being 0.42 gm. (urine, 0.34, feces, 0.08 gm.). Without intermission there followed another 3 day experiment in which milk was added to the diet bringing the daily calcium intake to 0.39 gm., the output being the same (urine, 0.28, feces, 0.11 gm.), so that equilibrium was established at this figure. Then meat (lean beef) was added to the diet with the result that the daily calcium intake was increased by 0.01 gm. and the output rose 0.16 gm. (urine, 0.36, feces, 0.19 gm.). This loss of body calcium following the addition of meat to the diet might have been thought to be due to the fact that the added meat gave to the diet as a whole an excess of acid-forming over base-forming mineral elements; but other data obtained in this laboratory (Sherman, Gillett, and Pope[4]) do not indicate any such marked relation between the preponderance of acid- or base-forming elements in the food and the amount of calcium needed for maintenance.

---

[4] Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 301.

[5] Sherman, H. C., Winters, J. C., and Phillips, V., *J. Biol. Chem.*, 1919, xxxix, 53.

TABLE I.*

Indicated Calcium Requirement for Maintenance per 70 Kilos of Body Weight per Day.

Experiment No.	Calcium.	Experiment No.	Calcium.	Experiment No.	Calcium.	Experiment No.	Calcium.
	gm.		*gm.*		*gm.*		*gm.*
1	0.29	25	0.64	49	0.39	73	0.50
2	0.28	26	0.44	50	0.61	74	0.60
3	0.82	27	0.56	51	0.43	75	0.50
4	0.42	28	0.70	52	0.58	76	0.41
5	0.35	29	0.54	53	0.53	77	0.37
6	0.66	30	0.53	54	0.53	78	0.42
7	0.46	31	0.39	55	0.58	79	0.40
8	0.66	32	0.49	56	0.30	80	0.40
9	0.61	33	0.55	57	0.29	81	0.39
10	0.65	34	0.48	58	0.40	82	0.42
11	0.78	35	0.35	59	0.28	83	0.39
12	0.54	36	0.28	60	0.28	84	0.33
13	0.61	37	0.30	61	0.27	85	0.45
14	0.61	38	0.27	62	0.29	86	0.33
15	0.49	39	0.32	63	0.30	87	0.50
16	0.40	40	0.34	64	0.60	88	0.37
17	0.42	41	0.29	65	0.55	89	0.54
18	0.44	42	0.46	66	0.59	90	0.40
19	0.33	43	0.42	67	0.54	91	0.31
20	0.41	44	0.41	68	0.55	92	0.44
21	0.35	45	0.43	69	0.47	93	0.35
22	0.40	46	0.46	70	0.46	94	0.29
23	0.40	47	0.50	71	0.40	95	0.33
24	0.53	48	0.47	72	0.55	96	0.38
						97	0.34
Average							0.45

* Experiments 1 and 2, Bertram, J., *Z. Biol.*, 1878, xiv, 354. No. 3, Renvall, G., *Skand. Arch. Physiol.*, 1904, xvi, 94. Nos. 4 and 5, von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211. No. 6, Holsti, Ö., *Skand. Arch. Physiol.*, 1910, xxiii, 143. Nos. 7 to 9, Sherman, Mettler, and Sinclair.[3] Nos. 10 to 15, Rose, A. R., and Sherman, not previously published. Nos. 16 to 34, Sherman, Gillett, and Pope.[4] Nos. 35 to 48, Sherman, Wheeler, and Yates.[4] Nos. 49 to 55, Sherman, Gillett, and Pope.[4] Nos. 56 to 58, Sherman, not previously published. Nos. 59 to 63, Sherman and Osterberg, not previously published. Nos. 64 and 65, Sherman and Winters.[4] Nos. 66 to 75, Sherman, Winters, and Phillips.[5] Nos. 76 to 97, Rose, M. S., *J. Biol. Chem.*, 1920, xli, 349, and unpublished data.

Subject R, weighing 80 kilos, took during five consecutive 3 day experiments without intermission a diet of bread, butter, and apples furnishing an average daily intake for each of the five periods of 0.23, 0.21, 0.20, 0.21, 0.21 gm., respectively. The corresponding output for each experimental period was: in urine, 0.11, 0.17, 0.16, 0.18, 0.18; in feces, 0.21, 0.15, 0.15, 0.16, 0.16 gm. of calcium per day (Experiments 59 to 63 in Table I).

For purposes of comparison and discussion the data of all available experiments which seem to be quantitatively comparable (including, through the courtesy of Professor M. S. Rose, some unpublished experiments recently performed in her laboratory) have been computed to a uniform basis of daily calcium output per 70 kilos of body weight and the results brought together in Table I. Since calcium leaves the body so largely by way of the intestine, the error which may occur in the separation of the feces belonging to successive experimental periods is likely in the case of calcium to be more significant than in the case of phosphorus, and relatively much greater than in the case of nitrogen. Knowing by experience how readily a part of the calcium output properly belonging to an experimental period may thus be carried over into the one following, the writer has had this in mind in compiling the data of other investigators as well as those from his own laboratory and has sometimes treated two successive periods as one when convinced by examination of the protocols that a more trustworthy result is thus obtained. It will be seen that there are included in the table the data of 97 experiments (experimental periods of 3 to 8 days) showing an extreme range of 0.27 to 0.82 gm. and an average of 0.45 gm. per 70 kilos of body weight per day.

In the discussion of an analogous compilation of the data of experiments upon protein metabolism,[6] it was pointed out that in an appreciable proportion of those experiments the protein requirement was probably overestimated, largely because, influenced by previous overestimates of the amount of protein needed in nutrition, the experimenters did not reduce the protein intake to a low enough figure and for a long enough time really to determine the minimum amount on which equilibrium could have

[6] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

been maintained. So far as this source of error is concerned the tendency in the study of calcium requirement has doubtless more often been in the other direction. In the past the calcium requirement of the body was underestimated. Experiments made to test calcium requirement have sometimes involved the use of diets furnishing so little calcium that the output (although greater than the intake) has been depressed below the point at which equilibrium could actually be maintained. Largely for this reason it is probable that some of the data for indicated calcium requirement in Table I, based as they are on the data of output when the intake was somewhat insufficient, are appreciably below the true requirements of the respective subjects. Doubtless there are other cases in which the requirement was overestimated through the use of diets unnecessarily rich in calcium for the purpose of the test; but these appear to be fewer in number and the general average of 0.45 gm. of calcium (equivalent to 0.63 gm. of CaO) is probably not above the true requirement.

Having now on record about 100 cases each of reasonably comparable experiments designed to measure the maintenance requirements for protein, phosphorus, and calcium, respectively, in normal human nutrition, it is of interest to compare the variability of the individual observations in the three series of experiments (each representing the work of several different investigators) and the probable errors of the three mean results as computed by accepted statistical methods.

The 109 experiments upon protein requirement[6] show a mean of 44.4 gm., a standard deviation of 9.07 gm., a coefficient of variation of 21, and a probable error of the mean of ±0.58 gm.

In 95 experiments upon phosphorus requirement[7] the mean with its probable error is 0.88 ± 0.01 gm., the standard deviation is 0.15 gm., and the coefficient of variation is 17.

The 97 experiments upon calcium requirement tabulated above give a mean result of 0.45 gm. with a probable error of ±0.008 gm. and show a standard deviation of 0.12 gm. and a coefficient of variation of 27.

In each of the three cases the data are now sufficiently numerous and consistent so that the probable error of the mean is less than two one-hundredths of its value and therefore presumably as dependable as the accepted statements of average composition of even the most familiar of our staple foods.

---

[7] **Sherman, H. C.,** *J. Biol. Chem.*, 1920, xli, 173.

It will be noted also that the coefficient of variation has been found to be of the same order of magnitude in the experimental study of protein, of phosphorus, and of calcium requirements. If any significance is to be attached to the slightly higher coefficient of variation in the data for calcium over those for protein or for phosphorus, it must follow that the case of calcium is the one which would seem to call for the most liberal margin of intake over the estimated average maintenance requirement if individual variability is to be covered by an ample factor of safety. Apparently a large proportion of American dietaries are open to improvement in this respect.

Comparing the present average of 0.45 gm. per man per day as the indicated calcium requirement with the previously reported average of 44 gm. as an indicated protein requirement, and keeping in mind the fact that the figure for protein is more likely to be an overestimate than that for calcium, and that the relative probable error due to individual variations is very similar in the two cases, we are forced to conclude that a food supply, in order to furnish these essential nutrients in relative proportions corresponding to the needs of the body, should contain at least 1.0 gm. of calcium (or 1.4 gm. of CaO) for every 100 gm. of protein. In the large majority of American food supplies of families and larger groups, as indicated by dietary studies made before the war by the United States Department of Agriculture and the New York Association for Improving the Condition of the Poor, this has not been the case. It does not follow that the calcium content was necessarily too low in the majority of dietaries, but rather that the food supply furnished a much more liberal surplus of protein than of calcium. When the results of 224 presumably typical American dietary studies were calculated to the usual basis of nutrients per man per day the average protein content was found to be 106 gm., or 140 per cent above the indicated maintenance requirement of 44 gm., while the average calcium content was 0.74 gm., or 64 per cent above the estimated actual minimum of 0.45 gm. Probably more significant than the average content of all the dietaries is the proportion of cases falling below the estimated requirement, either as regards protein, or as regards calcium. Only one of the 224 cases studied showed less protein than the indicated requirement, while one in every six was below the indicated requirement in calcium. In other words, the comparison of dietary studies or records of actual food supplies with the laboratory evidence of nutritive requirement suggests that very many more American dietaries are deficient in quantity of calcium than are deficient in quantity of protein, though of course there may be many cases in which the kind of protein, or the intake of some particular amino-acid, is unsatisfactory. If, in all the dietaries in the above group of 224 which showed less than 3,000 calories per man per day, the amount of food had been increased up to that level, there would have been no case of protein deficiency as judged by this standard while by the same criterion about 7 per cent or one in every fourteen of the dietaries studied would still be deficient in calcium. There seems to be no room for doubt that more attention should be given to the calcium intake both in human nutrition and that of farm animals. So far as the requirement for calcium in itself is concerned the intake may be supplemented by purely mineral additions as when the animal feeder includes finely ground rock phosphate ("floats"), bone ash, or oyster shells in the rations which he provides. Similarly calcium carbonate or phosphate might be habitually added to human food, either separately or by mixing it with the table salt used in seasoning; but it would probably be more difficult to persuade people generally to make such additions than to teach a more liberal use of foods naturally rich in calcium, while the latter course has the added advantage that the foods whose larger use would be invoked to increase the calcium intake (notably milk in its various forms) are important sources of proteins of high nutritive efficiency and of the fat-soluble vitamine as well.

THE PRODUCTION OF ACETALDEHYDE BY CERTAIN PENTOSE-FERMENTING BACTERIA.*

By W. H. PETERSON and E. B. FRED.

(*From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.*)

(Received for publication, July 22, 1920.)

The search for intermediate products in the fermentation of the carbohydrates has occupied the attention of scientists for many years. This search has been pushed most vigorously in connection with the alcoholic fermentation by yeast. The investigations of Neuberg, Harden, and Kostytschew are most conspicuous and have resulted in a better understanding of the biochemical changes that occur. Neuberg's theory (1) that pyruvic acid is an intermediate product in alcoholic fermentation is now generally accepted. This theory rests mainly on indirect evidence, although Fernbach and Schoen (2) and Beijerinck and Folpmers (3) reported the production and isolation of pyruvic acid from fermenting sugar cultures, and Mazé and Ruot (4) claim to have obtained it by the fermentation of lactic acid with bacteria. Neuberg found that pyruvic acid is fermented by yeast with the production of carbon dioxide and acetaldehyde and he isolated and identified the acetaldehyde thus formed. Although acetaldehyde has been observed many times in different types of fermentation, until recently no conclusive evidence has been obtained to show that it is an intermediate product in sugar fermentations. The occurrence in wine was noted by Magnes-Lahens (5) in 1855 and since that time its presence and the factors related to its occurrence have been the subject of numerous investigations (6). Trillat (7) and his associates as a result of their investigations concluded that acetaldehyde was not an intermediate product in the fermentation of sugar but arose from a secondary oxidation of the ethyl alcohol.

Buchner, Langheld, and Skraup (8) obtained small quantities of aldehyde in the fermentation of sugars with yeast juice and likewise concluded that it was derived from ethyl alcohol by oxidation.

Kostytschew (9) on the other hand regarded acetaldehyde as an intermediate product and by the addition of zinc chloride to a fermentation of

---

* This work was in part supported by a grant from the special research fund of the University of Wisconsin.

Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

glucose by yeast obtained enough aldehyde to prepare the *p*-nitrophenylhydrazone. The amount obtained was small and Kostytschew's conclusion that acetaldehyde is an intermediate product was vigorously assailed by Neuberg (10) and his coworkers.

In the fermentation of glucose by *Bacillus coli communis*, Grey (11) reported the production of a minute quantity of acetaldehyde. From the fermentation of 7.46 gm. of glucose he obtained 0.00234 gm. of acetaldehyde.

Numerous attempts have been made to increase the quantity of acetaldehyde by the addition of various substances. This was the object sought by Kostytschew with zinc chloride. In a later investigation Kostytschew (9) employed methylene blue to serve as an acceptor for hydrogen and thus to check the reduction of acetaldehyde to alcohol. The result was an increase in the yield of acetaldehyde. Later Palladin and Sabinin (12) found that methylene blue enhanced the production of acetaldehyde from lactic acid.

Müller-Thurgau and Osterwalder (13) increased the quantity of acetaldehyde formed in wine by adding small quantities of potassium *m*-bisulfite at varying intervals during the fermentation. Similar experiments with sucrose and glucose gave from 0.350 to 1.180 gm. of aldehyde per 100 gm. of sugar fermented. Previously Passerini (14) had obtained an increase in the aldehyde content of wines by the addition of bisulfite.

In an investigation of the factors that influence the formation and disappearance of acetaldehyde from wines Laborde (6) confirmed the observations of earlier investigators, that acetaldehyde may originate from the oxidation of alcohol or be formed as an intermediate product in the breaking down of sugar. His results showed a marked increase in the production of acetaldehyde when sulfurous acid was added to the fermenting solutions.

It was not until Neuberg and his coworkers (1, 15) employed a more suitable fixative that acetaldehyde was obtained in large amounts, and thus established the relation of acetaldehyde to the fermentation of sugar. With sodium sulfite as a fixative, he obtained acetaldehyde equivalent to 17.95 per cent of the sugar fermented. The aldehyde was determined by three different methods and was also isolated and identified by its hydrazone with *p*-nitrophenylhydrazine. The aldehyde-sulfite compound was isolated and identified as $(CH_3CHOH.SO_3Na)_2+H_2O$. These results present convincing and conclusive evidence for the existence of acetaldehyde as a product in the fermentation of sugars by yeast.

In a later publication, Neuberg and Nord (16) extended the work to the fermentation of sugars by bacteria and found that from 0.0014 to 0.2532 gm. of aldehyde formed in 100 cc. of culture containing 2 per cent of glucose. Indications of aldehyde formation by bacteria have been observed by others.

Harding and Ostenberg (17), in a study of the color changes produced by bacteria on the fuchsin-sulfite media of Endo, assumed that aldehyde is produced from the carbohydrate. At first the aldehyde combines with the sulfite and is thus prevented from oxidizing to acid, but later when the sulfite is used up additional aldehyde undergoes oxidation to acid and in this way decolorizes the medium. They suggest that aldehyde formation may be used as a basis for the differentiation of colon and typhoid organisms.

De Bord (18) reported that in the breaking down of glucose and lactose by many kinds of bacteria, aldehyde is formed. The organisms were grown in tube cultures and aldehyde formation was tested with Schiff's fuchsin reagent. No evidence is presented to prove the identity of the aldehyde. Since Schiff's reagent is affected by many substances other than aldehyde, de Bord's results are by no means conclusive.

Buchner and Meisenheimer (19) suggested that in a fermentation yielding butyric acid, butyl alcohol, and other substances acetaldehyde is first produced. Harden (20), in a detailed study of the action of *Bacillus coli* and similar organisms on carbohydrates, advanced the theory that the carbohydrate is decomposed into an intermediate substance which, in turn, gives rise to formic acid and acetaldehyde. The formic acid later is converted into CO_2 and hydrogen.

The work described in this paper was planned primarily to study acetaldehyde formation by pentose-fermenting bacteria in the breaking down of both hexose and pentose sugars. Three widely separated types of bacteria were selected for this investigation. The first, to which has been given the laboratory number 26, was isolated from sunflower silage and apparently belongs to the colon-aerogenes group. Its biochemical and cultural characteristics will be described in a later paper. It ferments all the common sugars and in addition attacks vigorously the pentoses, xylose and arabinose. The second organism used was *Bacillus acetoethylicum*, isolated and described by Northrop and his associates (21). The third group of bacteria was the pentose fermenter *Lactobacillus pentoaceticus, n. sp.*, whose isolation and fermentation characteristics have been described in a previous publication (22).

EXPERIMENTAL.

In general the carbohydrates were fermented in a yeast water medium with and without peptone, to which an excess of calcium carbonate was added. In order to hold the aldehyde, the fixative calcium sulfite or sodium sulfite was used. The calcium sulfite was added to the yeast water before sterilization, and in the experiments where sodium sulfite was used a 20 per cent aqueous solution of the sulfite was sterilized and the desired amount of this solution added to the culture. The calcium sulfite was freshly prepared from sodium sulfite and calcium chloride, air-dried, and used as the hydrated salt $CaSO_3.2\ H_2O$. The sodium sulfite contained a considerable amount of sodium sulfate and hence the figures given in the tables represent only approximately the concentration of sulfite in the medium.

The alcohol, volatile acid, and non-volatile acid were determined as described in previous publications. Acetaldehyde was determined according to the method developed by Neuberg and Nord (16). 100 cc. of culture were cooled with ice and an amount of barium chloride sufficient to precipitate the sulfite was added. After the addition of 10 gm. of calcium carbonate the flask was placed in an oil bath and joined to a long Allihn condenser. The delivery end of the condenser was connected to a thick walled Pyrex flask packed in a freezing mixture of ice and salt. A thistle tube partly filled with water was attached to the Pyrex flask to serve as an escape tube for the air in the apparatus. After about one-third of the contents of the flask had been driven over, steam was passed in and the distillation continued until about 400 cc. had collected in the receiving flask. The distillate was made up to a definite volume and the aldehyde in an aliquot determined by the iodine titration method (23).

Trial determinations with dry redistilled acetaldehyde (boiling point 20–21°C.) to which the above reagents had been added gave a recovery of from 98 to 100 per cent.

Acetaldehyde Production by Culture 26.

Culture 26 ferments the pentoses, xylose and arabinose, vigorously and tolerates a high degree of alkalinity. Since the culture solutions containing sodium sulfite are distinctly alkaline (pH 8.0) this organism is well adapted to the conditions existing in a sulfite medium. Three flasks of 2 per cent glucose-yeast water medium were inoculated with this organism and, after 24 hours of fermentation, 10, 20, and 30 cc. of a 20 per cent solution of sodium sulfite were added to the respective flasks. Here the concentration of the sulfite was 0.5, 1.0, and 1.5 per cent as calculated on the original volume. At frequent intervals the cultures were tested for aldehyde by means of sodium nitroprusside and diethylamine

(Simon's test as modified by Rimini (24)). The most satisfactory tests were obtained by mixing 5 cc. of culture and 0.5 cc. of a fresh 4 per cent solution of prusside, and carefully adding 5 to 10 drops of a 5 per cent solution of the amine so as to form a layer on top. After about 20 to 30 seconds, a sharp blue zone forms where the two liquids come together. If the test-tube is held against a white background, this zone becomes more distinct. In the presence of much aldehyde the blue color extends through the entire solution when the tube is shaken. No aldehyde could be detected until the 3rd day, when a slight test was found in Flask 3. After 5 days all the cultures gave a distinct blue color with these reagents. The intensity of color increased with the age of the culture until after 8 days a deep blue color extending to the bottom of the tube was obtained. In all the experiments that follow, the formation of aldehyde was traced by means of the Rimini test. The distillate obtained in the quantitative procedure was also tested for aldehyde by the Rimini test and in some cases with Schiff's reagent. A typical example of the results obtained from qualitative tests is given in Table I. Although these tests may be made on the cultures direct they are far better when used on the distillate. The amount of aldehyde formed was determined at the end of 7 days and again at the end of 12 days. On the second analysis a small increase was found in only one flask, No. 3. From this and other data (see Table II), it is apparent that aldehyde production is most active between the 2nd and 5th days, a period which

TABLE I.

Qualitative Tests for Acetaldehyde Obtained at Different Stages in Fermentation.

Flask No.	Sodium sulfite.	On culture.				On distillate of the culture.
		3 days.	5 days.	7 days.	10 days.	10 to 12 days.
	per cent					
1	1	None.	Fair.	Good.	Strong.	Strong.
2	2	"	Slight.	"	"	"
3	3	"	"	Fair.	"	"

corresponds approximately to the time of greatest activity in the fermentation. The quantity of aldehyde fixed increases with the amount of sulfite present (see Table III); *e.g.*, with 1 per cent of sulfite the aldehyde fixed is 0.028 gm. per 100 cc. of culture and with 3 per cent of sulfite this has increased to 0.077 gm. At the end of the fermentation period, the control, Culture 1, to which no sulfite was added, and Cultures 3 and 4 were analyzed for alcohol, volatile acid, and non-volatile acid in addition to aldehyde. A high alcohol and low acid production is the normal type of fermentation with Culture 26. When sulfite is introduced, some of the intermediate compound acetaldehyde is fixed and the production of alcohol is decreased from 0.281 to 0.139 gm. per 100 cc. of culture. Coincident with the aldehyde and low alcohol production there is a rise in the volatile acid from 0.036 gm. in the control to 0.148 gm. in the flask containing 3 per cent of sodium sulfite. The volatile acid is evidently an oxidation product with which should be paired some reduction product, unless the hydrogen which is one of the gases formed under these conditions can account in some type of reaction for the oxygen used in the oxidation. Insufficient data exist to determine whether there is any relation between the hydrogen and the volatile acid. The complete data are given in Table III.

TABLE II.

Rate of Acetaldehyde Production by the Fermentation of Glucose. Calculated for 100 Cc. of Culture.

Age of culture.	Acetaldehyde.	Ethyl alcohol.	Volatile acid as acetic.
days	*gm.*	*gm.*	*gm.*
2	0.025		
4	0.046		
6	0.053		
11	0.065		
15	0.063		
20	0.068	0.075	0.232

Unfermented sugar 1.65 per cent.

In order to have sufficient aldehyde to form the hydrazone with *p*-nitrophenylhydrazine a 2,000 cc. culture was prepared

The culture contained 3 per cent of glucose and 4 per cent of sodium sulfite. Aliquots were withdrawn at different intervals during the fermentation and the aldehyde was determined, as shown from the figures of Table II. The aldehyde content increases steadily up to the final analysis but most of it is formed by the end of 6 days. The low alcohol and high volatile acid production noted in Table III were repeated in this experiment.

The unfermented sugar was determined by the Ost method (25), since the Ost copper solution is not reduced to any extent by acetaldehyde as is the case with Fehling's solution. Glucose may, therefore, be determined in the presence of acetaldehyde by this method. The unfermented glucose amounted to 1.65 per cent which indicates that about one-half the glucose had been fermented. The data are given in Table II.

TABLE III.

Acetaldehyde Production from Glucose.

Calculated for 100 Cc. of Culture.

Flask No.	Sodium sulfite in culture.	Age of culture.	Acet-aldehyde.	Ethyl alcohol.	Volatile acid as acetic.	Non-volatile acid as lactic.
	per cent	*days*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.0	12	0.006	0.281	0.036	0.000
2	1.0	7	0.028			
3	2.0	7	0.061			
3	2.0	12	0.045		0.112	0.010
4	3.0	7	0.061			
4	3.0	12	0.077	0.139	0.148	0.078

The remainder of the culture was treated with the required amount of barium chloride and the precipitated barium sulfite removed by filtration. To prevent loss of aldehyde during the filtration, this operation was carried on in a large refrigerator and the receiving flask was surrounded by a mixture of ice and salt. The clear solution thus obtained could be distilled without the foaming that occurs when the barium sulfite is not removed. About 1,500 cc. of filtrate were distilled with steam until the last portions of the distillate gave no test for aldehyde. The distillate was redistilled and collected in a 3 per cent solution of *p*-nitrophenylhydrazine dissolved in 50 per cent acetic acid.

About one and one-half times the theoretical quantity of hydrazine required to combine with the aldehyde was used. When the first few drops of distillate reached the receiving flask, a curdy yellow precipitate of acetaldehydehydrazone was formed. The distillation was continued until about 300 cc. of distillate had been collected. The vapors passing over at this time gave a negative Rimini test. After standing over night in the ice box, the hydrazone was filtered off with suction, washed with a little cold water, and dried over sulfuric acid. The melting point of the crystals was found to be 125°C. They were dissolved in 95 per cent alcohol and recrystallized. The melting point of the recrystallized hydrazone was found to be 128°C. A hydrazone prepared from acetaldehyde gave the same melting point. The melting point of acetaldehyde-*p*-nitrophenylhydrazine is given as 128.5°C.

TABLE IV.

Determination of Nitrogen in Acetaldehyde-p-Nitrophenylhydrazone.

Method.	No. of determinations.	Nitrogen.
		per cent
Kjeldahl, modified to include nitrates	5	10.6 to 21.2
Milbauer	1	3.8
" modified	3	23.15 to 23.37
Dumas combustion	1	23.8
Theory for $C_8H_9N_3O_2$	1	23.46

In order to check the evidence obtained from the melting point, the nitrogen content of the hydrazone was determined by the Dumas combustion and a modified Kjeldahl procedure. At first much difficulty was experienced in determining the percentage of nitrogen in the hydrazone by the Kjeldahl method. By increasing the amount of potassium persulfate, the time of digestion, and by adding potassium permanganate, Milbauer's (26) modified Kjeldahl method gave results that checked with the Dumas combustion method and with theory. To indicate how the figures varied, a summary of the results obtained are given in Table IV. The melting point and nitrogen content of the hydrazone and the qualitative color tests establish, without doubt, the fact that acetaldehyde is formed.

Acetaldehyde from Xylose.

Since Culture 26 is a rapid pentose fermenter, it seemed probable that acetaldehyde would be formed in the fermentation of xylose by this organism. Experiments were made with test-tube cultures to which were added varying amounts of sodium sulfite. In nearly every case, a strong test for aldehyde was noted. A flask containing 300 cc. of 2 per cent xylose-yeast water medium was inoculated and 15 cc. of a 20 per cent sodium sulfite solution were added at the time of inoculation. At the end of 1, 2, and 6 days, additional portions of sulfite were added until the final concentration of sulfite reached 3.5 per cent. At the end of 5 days, a 50 cc. aliquot was taken and the acetaldehyde determined. Successive determinations were made at the end of 7, 11, and 15 days. Alcohol, volatile acid, and non-volatile acid were also determined at the time of the last analysis. As was found in the fermentation of glucose, the aldehyde continues to increase to the end of the fermentation. The quantity of aldehyde is more than double that formed from glucose, and is the maximum amount obtained in any of the experiments. As already noted in the case of glucose, an unusually high production of acetic acid was found. The data are given in Table V.

TABLE V.

Production of Acetaldehyde by the Fermentation of Xylose.
Calculated for 100 Cc. of Culture.

Age of culture.	Acetaldehyde.	Ethyl alcohol.	Volatile acid as acetic.	Non-volatile acid as lactic.
days	*gm.*	*gm.*	*gm.*	*gm.*
5	0.088			
7	0.102			
11	0.147			
15	0.157	0.074	0.486	0.150

Unfermented sugar 0.938 gm.

Acetaldehyde Production by Bacillus acetoethylicum.

This organism possesses the power of fermenting a wide range of carbohydrates among which is xylose. One of the conspicuous products of its activity is acetone. Under suitable conditions this represents about 8 to 10 per cent of the carbohydrate in the culture (21). Besides acetone, formic and acetic acids, ethyl alcohol, lactic acid, and carbon dioxide are produced (27). The formation of acetaldehyde is of special interest in relation to the production of acetone. The reactions whereby the acetone is formed are not clearly understood but in all probability its production is associated with the condensation of the acetaldehyde and subsequent processes of oxidation and decarboxylation. As a tentative suggestion these changes may be represented as follows:

TABLE VI.

Production of Acetaldehyde in the Fermentation of Starch by Bacillus acetoethylicum in the Presence of Sulfites.

Calculated for 100 Cc. of Culture.

Experiment.	Flask No.	Sulfite.	Amount of sulfite.	Age of culture.	Acetaldehyde.
			per cent	*days*	*gm.*
A	1	Na_2SO_3	1.0*	14	0.018
	2		2.0*	14	0.013
	3		3.0*	15	0.020
B	1	Na_2SO_3	1.0*	12	0.010
	2		2.0*	12	0.027
	3		3.0*		–†
C	1	$CaSO_3.2H_2O$	1.0‡	7	0.012
				10	0.028
	2		2.0‡	9	0.023
				13	0.042
	3		3.0‡	13	0.000

* Anhydrous Na_2SO_3.
† Negative qualitative test, not analyzed.
‡ Calculated as $CaSO_3$.

$$2CH_3.CHO = CH_3CHOH.CH_2CHO$$
$$CH_3CHOH.CH_2CHO + O_2 = CH_3-CO.CH_2COOH + H_2O$$
$$CH_3CO.CH_2COOH = CH_3.CO.CH_3 + CO_2$$

Reactions similar to the above are known to take place in fermentations and have been proposed by Neuberg and others (28) to explain the formation of different products. Reilly and his associates (29) have proposed somewhat similar reactions for the production of acetone by *Bacillus granulobacter pectinovorum.* The production of 2.3–butylene glycol and acetylmethylcarbinol was explained by Harden and Norris (30) on a similar basis. In their work on the production of butyric acid by *Bacillus butylicus* from glucose and glycerol, Buchner and Meisenheimer (19) suggested that acetaldehyde was first formed from the glycerol and was then condensed to aldol and subsequently reduced or oxidized to form butyl alcohol and butyric acid, respectively. The importance of these balanced processes of reduction and oxidation in the organism has been emphasized by Palladin in a recent publication. Neuberg's work on the decarboxylation of pyruvic acid is so well known as scarcely to need mention.

In the experiments with *Bacillus acetoethylicum,* three series of flasks were prepared at different times with varying amounts of sodium or calcium sulfite as the fixative. The results obtained are given in Table VI, and are of the same general character as those obtained with the previous organism although only about one-half as much aldehyde was fixed. An increase of aldehyde results from increasing the fixative up to the limit of tolerance for sulfite by the bacteria. Calcium sulfite was rather more effective than sodium sulfite, as about one-half more aldehyde was obtained from the cultures where calcium sulfite was used.

Acetaldehyde from Xylose by Bacillus acetoethylicum.

In this experiment a peptone-phosphate medium containing 2.0 per cent of xylose was used. In one culture, sodium sulfite was used as the fixative agent and in another, calcium sulfite.

TABLE VII.

Production of Acetaldehyde in the Fermentation of Xylose by Bacillus acetoethylicum.

Calculated for 100 Cc. of Culture.

Sulfite.	Amount of sulfite.	Age of culture.	Acetaldehyde.
	per cent	*days*	*gm.*
Na_2SO_3	2.0	8	0.030
Na_2SO_3	2.0	12	0.079
$CaSO_3.2H_2O$	1.5*	12	0.011

* Calculated as $CaSO_3$.

As the sodium sulfite contained considerably less sodium sulfate than that used in the other experiments, the amount added was correspondingly less. The cultures were analyzed at varying intervals during the fermentation and were found to contain a much larger amount of aldehyde than was obtained from the glucose cultures. Similar high yields of acetaldehyde from xylose have already been noted in the fermentation of xylose by Culture 26. Sodium sulfite proved far more efficient as a fixative of aldehyde than calcium sulfite. The data are given in Table VII.

Acetaldehyde Production by Lactobacillus pentoaceticus.

This group of bacteria is rather sensitive to alkali, as was discovered from the results of neutralizing actively fermenting cultures. If, inadvertently, a slight excess of alkali was added to the culture, growth ceased and was not resumed until a small quantity of acid was added. Therefore they do not lend themselves readily to experimentation in a sulfite medium. In the first series, a medium containing 2 per cent of glucose in yeast water was inoculated with the pentose fermenter No. 41-11 and, after 24 hours, enough sterilized sodium sulfite was added to give a concentraton of 1 and 2 per cent respectively in the two flasks.

TABLE VIII.

Production of Acetaldehyde by Lactobacillus pentoaceticus.
Calculated for 100 Cc. of Culture.

Flask No.	Sulfite in culture.	Age of culture.	Acetaldehyde.
	gm.	*days*	*gm.*
2	1.0*	14	0.029
3	2.0*	13	0.017
4	0.5*	15	0.000
5†	1.0*	14	0.063
6	1.0‡	12	0.008
7	2.0‡	10	0.016

* Sodium sulfite.

† Carbon dioxide produced was 0.025 gm.

‡ Calcium sulfite.

In a second series, the same percentage of calcium sulfite was added as a fixative. Direct qualitative tests on the culture showed only traces of aldehyde present. The strongest tests were obtained from the calcium sulfite series but the tests were only fair. After from 10 to 14 days, the cultures were analyzed for aldehyde, alcohol, volatile acid, and in a few cases for non-volatile acid. Only small quantities of these products were obtained. The carbon dioxide evolved from one of the flasks, No. 5, was determined and only 0.025 gm. per 100 cc. of culture was found. These results, coupled with strong reduction tests for glucose, showed that but little fermentation had taken place. The cause for this is probably due to the alkalinity of the sulfite medium or the toxic action of the sulfite on the bacteria. The data obtained for aldehyde are given in Table VIII.

The Fermentation of Pyruvic Acid.

According to Neuberg's theory, pyruvic acid is one of the intermediate products in alcoholic fermentation. The pyruvic acid then undergoes decarboxylation and acetaldehyde results. In the presence of sodium sulfite, Neuberg and Reinfurth (1) obtained 14 per cent of the theoretical yield of acetaldehyde, which would result if all the pyruvic acid had been completely fermented. The pentose-fermenting bacteria, employed in this study, ferment pyruvic acid to a greater or less extent. This has been shown in several ways: first, by the fermentation of the sodium salt of the acid and titration of the sodium bicarbonate formed; second, by the addition of dilute solutions of pyruvic acid to the fermenting culture and determination of the destruction of the free acid.

In the first experiment a 2 per cent solution of pyruvic acid was prepared, neutralized with solid sodium hydroxide, and then diluted to 1 per cent by the addition of double strength yeast water. This medium was then sterilized by passing it through a Pasteur-Chamberland filter, since a destruction of the pyruvate results if the solution is sterilized by heat. The solution was pipetted into large test-tubes and incubated for 5 to 6 days to determine its sterility. If no growth took place, the tubes were then inoculated, and sterilized brom-cresol purple was added. After a few days, a film of bacteria appeared on the surface, gas was evolved, and the solution became strongly alkaline. The above evidences of growth were very marked in the case of Culture 26 and *Bacillus acetoethylicum*, but were slight in the tubes inoculated with *Lactobacillus pentoaceticus*. The slight fermentation with the last named organism is probably due to its sensitiveness toward alkali. It is essentially an acid producer and does not withstand much alkali. The conclusions drawn from the appearance of the tubes during the incubation are borne out by the titrations at the end of the fermentation. If we judge from the alkali developed, about one-half the pyru-

TABLE IX.

Fermentation of Sodium Pyruvate by Pentose-Fermenting Bacteria.

Organism.	Tube No.	0.1 N sodium pyruvate present.	0.1 N alkali formed.
		cc.	*cc.*
Control	1	11.0	0.25
	2	11.0	0.60
No. 26	1	11.0	5.35
	2	11.0	6.60
B. acetoethylicum	1	11.0	1.50
	2	11.0	2.20
Lactobacillus pentoaceticus, No. 41-11	1	11.0	0.65
	2	11.0	1.22

In Tubes 1, pyruvic acid prepared in the laboratory was used.
In Tubes 2, pyruvic acid prepared by Eastman Company was used.

TABLE X.

Fermentation of Pyruvic Acid.

Tube No.	0.1 N acid.		
	Control.	Culture 26.	Culture 41-11.
	cc.	*cc.*	*cc.*
1	3.50	0.15	2.15
2	3.90		3.10
3	4.30	0.20	3.30
4	4.70	0.20	3.70
5	5.10	1.60	4.00

vate had been fermented by No. 26, about one-seventh by *Bacillus acetoethylicum*, and only a slight amount in the case of *Lactobacillus pentoaceticus*. The titration data are given in Table IX.

In a second experiment two series of large test-tubes containing varying amounts of yeast water were used. Sterilized pyruvic acid was added to these tubes from time to time and at the end of the incubation the unfermented acid was determined by titration. One series was left uninoculated and served as controls, a second series was inoculated with Culture 26, and a third with the pentose fermenter No. 41-11. Almost complete destruction of pyruvic acid resulted in the tubes inoculated with Culture 26 while only a small decrease of acid occurred in the tubes inoculated with Culture 41-11. The data obtained from the titrations are given in Table X. These two experiments showed that pyruvic acid and its sodium salt could be fermented.

An attempt was therefore made to ferment sodium pyruvate in the presence of sodium sulfite. To 100 cc. of 1 N pyruvic acid were added 80 cc. of 1 N sodium carbonate and 20 cc. of 1 N sodium sulfite, and this solution was made up to 1,000 cc. with yeast water. This medium is then equivalent to a 0.1 N solution of pyruvic acid and contains the sulfite in the form of the acid sulfite. It was sterilized by passing through a Chamberland filter and 18 cc. portions were pipetted into sterile fermentation tubes. After the tubes were incubated for a few days and no growth developed, they were inoculated in duplicate with Cultures 26, 41-11, and 118-8. One tube was left uninoculated as a control. A second series was set up where twice as much sulfite was added to the medium. The day after inoculation about 0.5 cc. of gas had accumulated in the tubes inoculated with Culture 26, and after 4 days this amounted to about 4 cc. The amount of gas collected in the tubes inoculated with Cultures 41-11 and 118-8 was never more than 1 cc. The small amount of gas accumulating is what would be expected since sodium bicarbonate is formed by the fermentation of sodium pyruvate. At the end of the fermentation period the carbon dioxide in the tubes was absorbed with sodium hydroxide. In the tubes inoculated with Culture 26 about one-half the gas remained unabsorbed and gave a test for hydrogen with a lighted splinter. The cultures were analyzed for their content of carbon dioxide by means of the Van Slyke apparatus and the data thus obtained are given in Table XI. In all the cultures the production of carbon dioxide is clearly indicated; the maximum was noted in the case of Culture 26. If the carbon dioxide found originated entirely by the decarboxylation of the pyruvate, the possible maximum would be 0.080 gm. In Tube 2 of Series 1, 0.0604 gm. of CO_2 was found.

TABLE XI.

Fermentation of Sodium Pyruvate in the Presence of Sodium Sulfite.

Tube No.	Carbon dioxide produced by		
	Culture 26.	Culture 41-11.	Culture 118-8.
	gm.	*gm.*	*gm.*
Series 1.			
Control.	0.0016	0.0016	0.0016
1	0.0385	0.0172	0.0227
2	0.0604*	0.0174	0.0130
Series 2.			
Control.	0.0017	0.0017	
1	0.0457	0.0167	
2	0.0441		

* Mercury added to retain gas.

SUMMARY.

In the presence of a fixative, acetaldehyde is formed in the fermentation of carbohydrates by three types of pentose-fermenting bacteria: an organism related to the colon-aerogenes group, Culture 26; *Bacillus acetoethylicum;* and *Lactobacillus pentoaceticus.* Aldehyde is produced from glucose and starch but the maximum yield is obtained from xylose. The amount formed ranges from 0.020 to 0.157 gm. per 100 cc. of culture containing 2 per cent of the carbohydrate. The acetaldehyde is fixed in the process of fermentation by means of sodium sulfite or calcium sulfite. The quantity of aldehyde found is proportional to the amount of fixative used. Because of the alkalinity of sodium sulfite, the amount that can be added is limited. Sodium sulfite was more effective than calcium sulfite as a fixative for the aldehyde.

The production of acetaldehyde is correlated with the production of alcohol. An increase in the former results in a decrease in the latter. A high production of volatile acid is associated with the formation of acetaldehyde. These results emphasize the intimate relations between the balanced processes of reduction and oxidation that exist in a fermenting solution.

The production of acetaldehyde by the acetone-forming organism, *Bacillus acetoethylicum*, is of particular interest since it is possible that through the condensation of the aldehyde and subsequent processes of oxidation and decarboxylation the acetone is formed.

An attempt to correlate the production of acetaldehyde with Neuberg's theory, that pyruvic acid is an intermediate product in sugar fermentation, did not give conclusive results. Pyruvic acid and its sodium salt are fermented to a greater or less extent by all three types of bacteria. The aerogenes-like bacteria fermented it readily, whereas *Lactobacillus pentoaceticus* gave but a slight fermentation. Among the products formed are carbon dioxide, hydrogen, and volatile acid. These products were also formed when the pyruvic acid was fermented in the presence of sodium sulfite, but neither qualitative nor quantitative methods showed the presence of acetaldehyde.

BIBLIOGRAPHY.

1. Neuberg, C., and Reinfurth, E., *Biochem. Z.*, 1918, lxxxix, 365.
2. Fernbach, A., and Schoen, M., *Compt. rend. Acad.*, 1913, clvii, 1478; 1914, clviii, 1719.
3. Beijerinck, M. W., and Folpmers, T., *Koninkl. Akad. Wetensch.*, 1916, xviii, 1198, cited in Neuberg and Reinfurth (1), p. 370.
4. Mazé, P., and Ruot, M., *Compt. rend. Soc. biol.*, 1916, lxxix, 706; 1917, lxxx, 336.
5. Magnes-Lahens, P., *J. pharm. Chim.*, 1855, xxvii, 37.
6. Kayser, E., and Demolon, A., *Compt. rend. Acad.*, 1908, cxlvi, 783; 1909, cxlviii, 103. Roeser, *Ann. Inst. Pasteur*, 1893, vii, 41. Laborde, J., *Ann. Inst. Pasteur*, 1917, xxxi, 215.
7. Trillat, A., *Ann. Inst. Pasteur*, 1908, xxii, 704, 753, 876. Trillat and Sauton, *Ann. Inst. Pasteur*, 1910, xxiv, 296, 302, 310.
8. Buchner, E., Langheld, K., and Skraup, S., *Ber. chem. Ges.*, 1914, xlvii, 2550.

9. Kostytschew, S., *Z. physiol. Chem.*, 1912, lxxix, 130. Kostytschew, S., and Hübbenet, E., *Z. physiol. Chem.*, 1912, lxxix, 359. Kostytschew, S., *Z. physiol. Chem.*, 1913, lxxxiii, 93.
10. Neuberg, C., and Kerb, J., *Biochem. Z.*, 1912, xliii, 494; 1913–14, lviii, 158; 1914, lxiv, 251.
11. Grey, E. C., *Biochem. J.*, 1913, vii, 359.
12. Palladin, W., and Sabinin, D., *Biochem. J.*, 1916, x, 183.
13. Müller-Thurgau, H., and Osterwalder, A., *Landw. Jahrb. Schweiz*, 1915, xxix, 408; abstracted in *Chem. Abst.*, 1916, x, 2274.
14. Passerini, N., *Chem. Centr.*, 1906, lxxvii, pt. 2, 1280.
15. Neuberg, C., and Ehrlich, M., *Biochem. Z.*, 1920, ci, 239.
16. Neuberg, C., and Nord, F. F., *Biochem. Z.*, 1919, xcvi, 133, 158.
17. Harding, E. R., and Ostenberg, Z., *J. Infect. Dis.*, 1912, xi, 109.
18. de Bord, G. G., *J. Bact.*, 1917, ii, 309.
19. Buchner, E., and Meisenheimer, J., *Ber. chem. Ges.*, 1908, xli, 1410.
20. Harden, A., *J. Chem. Soc.*, 1901, lxxix, 610.
21. Northrop, J. H., Ashe, L. H., and Senior, J. K., *J. Biol. Chem.*, 1919, xxxix, 1.
22. Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, xxxix, 347.
23. von Fürth, O., and Charnass, D., *Biochem. Z.*, 1910, xxvi, 207.
24. Simon, L., *Compt. rend. Acad.*, 1897, cxxv, 1105. Rimini, E., *Chem. Centr.*, 1898, lxix, pt. 2, 277.
25. Browne, C. A., A handbook of sugar analysis, New York, 1912, 433. Neuberg, C., and Reinfurth, E., *Biochem. Z.*, 1918, xcii, 259.
26. Milbauer, J., *Z. anal. Chem.*, 1903, xlii, 725.
27. Arzberger, C. F., Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xliv, in press.
28. Neuberg, C., in Oppenheimer, C., Handbuch der Biochemie, suppl., Jena, 1913, 40.
29. Reilly, J., Hickinbottom, W. J., Henley, F. R., and Thaysen, A. C., *Biochem. J.*, 1920, xiv, 229.
30. Harden, A., and Norris, D., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv, 492.

THE ESTIMATION OF CHLORIDES IN BLOOD.

By VICTOR C. MYERS and JAMES J. SHORT.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.)

(Received for publication, July 31, 1920.)

Renewed interest in blood chlorides has been aroused by Allen,[1] who has endeavored to show that salt retention is the cause of pure hypertension, and also of the hypertension in many cases of kidney disease. The estimation of this constituent has been most often conducted on the plasma, rather than on whole blood, but there would now appear to be some question as to the reliability of plasma analysis in this connection unless the plasma is immediately separated from the corpuscles. In any case a significant change in the blood chlorides should definitely affect the chloride content of whole blood as well as that of the blood plasma. The practical use of various laboratory tests of diagnostic import has ordinarily kept pace with their reliability and simplicity. That the estimation of the blood chlorides has not received more clinical consideration must be ascribed either to lack of appreciation of the possible value of the test, or to the difficulties in technique. A method of chloride estimation is described below which we believe contributes to the simplicity and rapidity of the test, without impairing its accuracy.

Most American workers who have studied this question have employed the iodimetric method of McLean and Van Slyke,[2] or some modification of it. With their original method the blood proteins were removed with the aid of heat, acetic acid, magnesium sulfate, and blood-charcoal. The chlorides were then precipitated in acid solution with silver nitrate and the excess was titrated with iodine, using starch as an indicator. Owing to the difficulty in securing suitable blood-charcoal, Harding and Mason[3] modified the method by employing copper sulfate and alkali for the removal of the protein. Later in the same year Foster[4] utilized *m*-phosphoric acid to remedy the same difficulty. Almost simultaneously with the publication of the McLean and Van Slyke method, Myers and Fine[5] described a technique of chloride estimation and later reported a few observations on the blood chlorides,[6] although these were not then discussed. With this method the proteins were coagulated in the presence of five volumes of 0.01 N acetic acid with a total dilution of 1 to 10, the last trace of protein being removed with a few drops of colloidal iron. The chlorides were then titrated either according to the Volhard-Arnold method, using solutions one-tenth the strength of those employed for urine, or directly with the Mohr method in this practically neutral solution. The disadvantage of this method was in the difficulty of obtaining chloride-free colloidal iron. 3 years later Rappleye[7] likewise made use of the Volhard titration for the estimation of chlorides in blood plasma. Still later Van Slyke and Donleavy[8] greatly simplified the McLean-Van Slyke technique by introducing sufficient picric acid into the silver nitrate-nitric acid reagent to precipitate completely the proteins and chlorides in one operation. Although this method proved perfectly satisfactory for plasma, Austin and Van Slyke[9] later noted that it gave too high results with whole blood, apparently as the result of the silver being bound by some constituent in the corpuscles. To overcome this difficulty for whole blood they now first precipitate the proteins with saturated picric acid solution. After filtration the chlorides are then estimated essentially as in the McLean-Van Slyke method.

---

[1] Allen, F. M., *J. Am. Med. Assn.*, 1920, lxxiv, 652.

[2] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

---

[3] Harding, V. J., and Mason, E. H., *J. Biol. Chem.*, 1917, xxxi, 55.

[4] Foster, G. L., *J. Biol. Chem.*, 1917, xxxi, 483.

[5] Myers, V. C., and Fine, M. S., Chemical composition of the blood in health and disease, New York, 1915, 34. also Myers, V. C., *Post-Graduate*, 1915, xxx, 38.

[6] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xx, 391.

[7] Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

[8] Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

[9] Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 345.

In connection with the present study we have tried a number of methods of protein precipitation, including the tungstic acid method of Folin and Wu.[10] None of these has proved so satisfactory in our hands as the use of picric acid recommended by Van Slyke and Donleavy. Several years ago an attempt was made to utilize the picric acid filtrate employed in the estimation of sugar and creatinine for the chlorides, but at that time this method was not believed to be so satisfactory as the heat and acetic acid procedure. Our present observations, however, confirm the findings of Austin and Van Slyke as to the reliability of picric acid as a blood protein precipitant in the estimation of chlorides. Furthermore, they indicate that it makes little difference in the results obtained whether the blood is diluted 1 to 20 as in their technique, or 1 to 10 or 5. Since the 1 to 5 dilution is employed in the estimation of sugar[11] and creatinine,[12] the chloride estimation may be made on the same filtrate. We have preferred to adhere to the Volhard titration partly because this allows the use of the same solutions (diluted) employed in the Volhard-Harvey[13] method for urine, and partly because of the rather rapid deterioration of the starch indicator used in the iodimetric method. When the starch solution is fresh the end-point is very sharp, sharper than in the thiocyanate titration, although with a little experience the latter is readily recognized. Many workers have complained of difficulties with the silver chloride filtration. We believe that the quickest and most satisfactory method of removing this rather troublesome precipitate is with the aid of the centrifuge as originally suggested.[5]

To summarize the advantages of the technique described for blood chlorides, the estimation may be carried out: (1) on the same filtrate as used in the estimation of creatinine and sugar; (2) with the same reagents (diluted) as commonly employed for chloride estimation in urine (Volhard-Harvey method); and (3) considerable time may be saved by removing the troublesome silver chloride precipitate by centrifuging rather than by filtration.

---

[10] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

[11] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

[12] Myers, V. C., *J. Lab. and Clin. Med.*, 1920, v, 566.

[13] Harvey, S. C., *Arch. Int. Med.*, 1910, vi, 12.

Method for Chloride Estimation in Blood.

When blood chlorides only are to be estimated there are some advantages in employing the 1 to 10 dilution and this technique will be described first.

Solutions Required.

1. Silver nitrate of such strength that 1 cc. is equivalent to 1 mg. of sodium chloride, 2.904 gm. to 1,000 cc. It may be prepared by a 1 to 10 dilution of the silver nitrate employed in the Volhard-Harvey method[18] for urine.
2. Acid ferric alum indicator prepared by dissolving 100 gm. of crystalline ferric ammonium sulfate in 100 cc. of 25 per cent nitric acid and adding four parts of distilled water. This is one-fifth the strength employed for urine.
3. Ammonium thiocyanate of such strength that 2 cc. are the equivalent of 1 cc. of the above silver solution, or one-twentieth of the strength employed for urine. It contains approximately 0.65 gm. of the thiocyanate to 1,000 cc.

To simplify the test further it is possible to combine Solutions 1 and 2, two parts of No. 1 and one part of No. 2 being used with the 1 to 10 dilution of the blood, this combined solution being further diluted with one part of distilled water when the 1 to 5 blood dilution is employed.

3 cc. of whole blood (or plasma) are added to 27 cc. of distilled water in a 50 cc. centrifuge tube. About 0.5 gm. of dry picric acid is then added and the mixture stirred until protein precipitation is complete and the whole mixture turns a bright yellow color. The precipitate is now thrown down by centrifuging for a few minutes at moderate speed and the clear supernatant fluid decanted into a clean dry beaker. (If any particles remain suspended the fluid should be filtered.)

20 cc. are then pipetted into a clean dry 50 cc. centrifuge tube and 20 cc. of standard $AgNO_3$ solution, of such strength that 1 cc. is equivalent to 1 mg. of NaCl (Solution 1), and 10 cc. of the dilute acidified ferric alum indicator added (Solution 2). The contents are stirred to insure thorough mixing and the AgCl preprecipitate is thrown down in the centrifuge. The clear supernatant fluid is decanted into a clean, dry beaker and 20 cc. portions are pipetted into each of two small porcelain evaporating dishes for duplicate titrations.

The titration is made with ammonium thiocyanate solution of such strength that 2 cc. are equivalent to 1 cc. of the $AgNO_3$ solution (Solution 3). The end-point is definite and consists of the first permanent tinge of reddish brown which extends throughout the mixture. Some experience may be necessary before the end-point is always recognized, but thereafter there need be no difficulty in obtaining exact duplicate titrations. Passing the end-point by one drop will introduce an error ordinarily of about 0.5 per cent in estimating chlorides in 100 cc. of blood or plasma.

When it is desired to utilize the 1 to 5 picric acid filtrate employed for sugar and creatinine, 5 cc. (equivalent to 1 cc. of blood) are employed, instead of 10 cc., for a single determination. If this method is to be followed regularly it is desirable to dilute the indicator solution further by one-half. In this case 5 cc. of the filtrate are pipetted into a 25 or 50 cc. centrifuge tube and 10 cc. of both the standard silver nitrate and ferric alum indicator added (or 20 cc. of the mixture referred to under "Solutions required"). After centrifuging titration is carried out on 20 cc. of the supernatant fluid as described above.

The calculation is $10 - \left(\frac{\text{Titer}}{2} \times \frac{5}{4}\right) \times 100 =$ mg. of NaCl in 100 cc. of whole blood or plasma.

With the above method it has been found possible to determine the sodium chloride content of pure solutions with an error of less than 1 per cent, and sodium chloride added to blood may be recovered with a similar degree of accuracy. A parallel series of results obtained with the Austin-Van Slyke method and the method described is given in Table I. As will be noted there is very close agreement between the two methods. In the case of the method described it apparently makes little difference whether a dilution of 1 to 5 or 1 to 10 is employed. All the observations reported in the table were obtained on whole blood.

In his first paper dealing with the chlorides of the blood plasma McLean[14] considered the influence of the plasma being allowed to stand in contact with the cells. He concluded that the change took place very slowly and that it was necessary only to centrifuge within 2 to 3 hours to avoid the danger. In their first paper on the plasma bicarbonate Van Slyke and Cullen[15] called attention to the effect which carbonic acid changes in whole blood might have on the chloride content of the plasma, a loss in bicarbonate resulting in an increase in the plasma chloride. This observation has recently been confirmed and extended by Fridericia,[16] who states that estimation of the chlorides in the plasma or serum from blood which has been kept in open receivers must give too high results, because chlorides have passed into the plasma (or serum) on account of the decreasing CO_2 tension. McLean, Murray, and Henderson[17] have likewise considered this question in a preliminary way. Observations bearing on this question are given in Table II. In taking the control

---

[14] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212.

TABLE I.

Comparison of Results Obtained with Austin-Van Slyke Method and Method Described.

Specimen.	NaCl per 100 cc.			Remarks.
	Austin-Van Slyke method.	Method described.		
		1:10 dilution.	1:5 dilution.	
	mg.	*mg.*	*mg.*	
1. J. J.	456	456	462	Normal.
2. M. B.	487		487	"
3. S. D.	487	481	500	"
4. I. S.	475	462	481	"
5. W. S.	447	444	444	"
6. R. I.	462	462	467	"
7. D.	475		475	Essential hypertension.
8. R. K.	456	460	456	Slight nitrogen retention.
9. F. M.	587	584	584	Advanced nephritis.
10. J. C.	531	537	537	" "
11. M. D.	475		469	" "
Average	485		487	

---

[15] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

[16] Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.

[17] McLean, F. C., Murray, H. A., Jr., and Henderson, L. J., *Proc. Soc. Exp. Biol. and Med.*, May 22, 1920.

specimens of blood under oil the technique of Van Slyke and Cullen[15] was followed. The subjects were normal, two being men and two women. In general it will be noted that standing increased the chloride content of the plasma. This being the case, results obtained on whole blood would appear to be more trustworthy than those obtained on plasma. Whole blood ordinarily contains about 100 mg. less NaCl per 100 cc. than plasma.

TABLE II.

Influence of Standing on Plasma Chloride.

Subject.	Chloride content of whole blood (NaCl per 100 cc.).	Chloride content of blood plasma (NaCl per 100 cc.).			
		Taken under oil and separated immediately.	Taken in open container and separated immediately.	Taken in open container and separated later.	
				Time.	NaCl.
	mg.	*mg.*	*mg.*	*hrs.*	*mg.*
1. J. S.	506	600	612	18	669
2. L. J.	475	587	644	2	612
3. H. C.	538	644	638	2	650
4. F. T.	538	612*		24	644

* After slow aeration for 5 min. the figure was 616 mg.

SUMMARY.

A method of chloride estimation in blood is described, in which the same picric acid filtrate employed for the estimation of sugar and creatinine, and the same solutions (diluted) ordinarily employed for the estimation of chlorides in urine are utilized.

RAPID COLORIMETRIC METHODS FOR THE DETERMINATION OF PHOSPHORUS IN URINE AND BLOOD.

By RICHARD D. BELL and EDWARD A. DOISY.

(From the Biochemical Laboratories of Harvard Medical School, Boston, and Washington University School of Medicine, St. Louis.)

(Received for publication, July 29, 1920.)

Several years ago, Taylor and Miller (1914) described a method for the determination of phosphates depending upon precipitation as ammonium phosphomolybdate and colorimetric estimation of the precipitated molybdenum by reduction with phenylhydrazine. Although this method has been quite extensively used, and other reducing agents have been proposed, the observation does not seem to have been made previously that certain reducing agents will reduce phosphomolybdic acid without affecting molybdic acid. By taking advantage of this selective reduction, the molybdenum present as phosphomolybdic acid can be colorimetrically determined in the presence of an excess of molybdic acid, avoiding the necessity of isolating the ammonium phosphomolybdate.[1]

We have found that hydroxylamine and many phenols have this selective reducing action in weakly acid solution at room temperature and we have selected hydroquinone as the most suitable reducing reagent. The color produced by the reduction of the phosphomolybdic acid by the hydroquinone is intensified by making the solution alkaline and a greenish shade due to the presence of quinone is removed by the addition of sulfite. The alkali (carbonate) and the sulfite are added in one solution.

---

[1] After this paper was prepared for publication, we were shown the proof of a paper recently published by Dr. Wu in this *Journal*, in which he refers to this specific reduction and proposes a tentative method for the determination of phosphate. While we knew that Dr. Wu was investigating phosphotungstic and phosphomolybdic acids, we were unacquainted with the details of his work and have derived no information from it. We demonstrated the reduction of phosphomolybdic acid and the non-reduction of molybdic acid by hydroxylamine in the presence of Dr. Wu several months before his paper was sent in for publication and stated that we intended to develop methods for the determination of phosphate on this basis. We, therefore, do not feel bound by his implied reservation of this field.

If hydroquinone is added to a weakly acid solution of molybdic acid, no phosphate being present, the mixture will be perfectly colorless when treated with alkaline sulfite, but, if phosphate is present, phosphomolybdic acid is formed and reduced giving a blue color. As little as 0.005 mg. of phosphorus as phosphate gives a distinct blue color in a volume of 100 cc. The reaction is the same as in the phenol determination of Folin and Denis (1915), except that in our case there is an excess of phenol, and in theirs an excess of phosphomolybdic acid.

In the actual determination, the solution to be determined is placed in one volumetric flask and a standard solution of potassium phosphate in another. Distilled (phosphate-free) water is added to make both solutions to the same volume, and molybdic acid and hydroquinone solutions are added to both. After standing 5 minutes, the alkaline sulfite solution is added, the flasks are made up to volume, and the colors compared in the colorimeter in the usual manner after further standing of 5 to 10 minutes. It is possible that not all the phosphomolybdic acid is reduced by this process, but the color is strictly proportional to the amount of phosphate present, over a wide range of concentration. The unknown and the standard should have nearly the same acidity, volume, and temperature during the reduction with hydroquinone in acid solution, since variation in these factors has some effect on the final depth of color.

We have developed methods for the determination of inorganic, organic, and total phosphorus in urine and inorganic and total acid-soluble phosphorus in whole blood, plasma, and serum, using the solutions given below.

Molybdic Acid Solution.—50 gm. of pure ammonium molybdate are dissolved without heat in 1 liter of phosphate-free normal sulfuric acid. 5 cc. of this solution, treated with an equal amount of the hydroquinone solution and, after standing 5 minutes, with 25 cc. of the carbonate-sulfite solution, should give an absolutely colorless mixture. If the ammonium molybdate contains phosphate, it may be purified as follows: 150 gm. of ammonium molybdate are dissolved in 1 liter of water and added to 375 cc. of concentrated nitric acid (specific gravity 1.42) which has been diluted to 1 liter. 200 gm. of ammonium nitrate are dissolved in this mixture and the whole is allowed to stand several days in a warm place. After the precipitated ammonium phosphomolybdate is filtered out, the filtrate is treated with two volumes of alcohol and then with ammonia until only faintly acid to litmus. The molybdate separates completely in a few minutes and is filtered out with suction, washed with 50 per cent alcohol, and dried.

This solution keeps indefinitely. The formation of a small amount of sediment does no harm; it may be removed by decantation.

Hydroquinone Solution.—20 gm. of pure hydroquinone are dissolved in 1 liter of phosphate-free water and 1 cc. of concentrated sulfuric acid is added. On standing, this solution becomes colored due to the formation of some quinone. A moderate amount of color does no harm as the quinone is reduced in the determination by the alkaline sulfite solution. The solution should be kept tightly stoppered to reduce the amount of oxidation by the air.

Carbonate-Sulfite Solution.—To 2,000 cc. of a 20 per cent solution of Na_2CO_3 are added 75 gm. of sodium sulfite dissolved in 500 cc. of water. The mixture is filtered. The presence of a moderate amount of phosphate in this solution does no harm since phosphomolybdates are not formed in alkaline solution.

Stock Solution of Monopotassium Phosphate.—Pure monopotassium phosphate is finely ground and exposed in a desiccator over sulfuric acid for several days. Of this dry preparation, 4.394 gm. are dissolved and made up to 1 liter with phosphate-free water in an accurate volumetric flask. 1 cc. of this solution contains 1 mg. of phosphorus. The solution should be preserved with chloroform and kept tightly stoppered.

Standard Phosphate Solution for Urine.—Of the above stock solution of phosphate, 50 cc. are accurately measured into a 500 cc. volumetric flask and made up to volume with phosphate-free water. This solution should also be preserved with chloroform. If the solution becomes turbid or if molds appear, a new solution should be made up. 5 cc. of this solution contain 0.5 mg. of phosphorus.

Dilute Standard Phosphate Solution for Blood.—5 cc. of the stock phosphate solution are made up to 1,000 cc. and preserved with chloroform in the same manner as the standard solution for urine. 5 cc. of this solution contain 0.025 mg. of phosphorus.

Urine.

Inorganic Phosphate.—1 to 5 cc. of urine, according to concentration, are accurately measured into a 100 cc. volumetric flask and 25 cc. of phosphate-free water added. In a similar flask are placed 5 cc. of the standard phosphate solution for urine, also with 25 cc. of water. To both flasks are added 5 cc. of the molybdic acid solution and 5 cc. of the hydroquinone solution. After standing 5 minutes, 25 cc. of the carbonate-sulfite solution are added and the flasks made up to volume and mixed. The solutions are read after 5 to 10 minutes. While the color is not absolutely permanent, we have found that the standard and the unknown fade at almost exactly the same rate, and that readings made at the end of an hour give the same result as those made at the end of 5 minutes. However, the solution in the cup of the colorimeter does not fade at the same rate as that in the flask, and both cups of the colorimeter should be filled at the same time whenever a comparison is to be made.

If the urine is read against the standard set at 20 mm. the calculation is as follows:

$$\frac{10}{\text{Reading} \times \text{cc. urine used}} = \text{Gm. inorganic P per liter}$$

If an insufficient amount of water is added to the urine before adding the molybdic acid, certain urinary constituents are precipitated by the phosphomolybdic acid leading to slightly high results.

Since 1 to 4 cc. of urine are diluted to 100 cc. during the determination, the color of the urinary pigments seldom interferes with the determination. We have even determined urines containing bile by the above process. If the color of the urine is troublesome, it may be removed by adsorption. For this purpose we have found certain samples of "activated carbon," prepared by the National Carbon Company of Cleveland, to be satisfactory.

A carbon to be used for this purpose should be carefully tested by comparing determinations on the standard solution with and without treatment with carbon, to insure that phosphate is neither added nor removed.

Total Phosphorus.—1 cc. of urine is measured with an Ostwald pipette into a hard glass test-tube and 6 to 8 drops of concentrated sulfuric acid, 1 cc. of concentrated nitric acid, and a piece of quartz are added. The tube is cautiously heated until nitrous fumes no longer come off and the remaining drops of sulfuric acid are colorless. Care should be taken to avoid evaporating to dryness or pronounced overheating which may cause loss of phosphoric acid.[2] The remaining few drops of sulfuric acid are transferred to a 100 cc. volumetric flask with about 25 cc. of phosphate-free water. Into a similar volumetric flask, containing 4 to 6 drops of concentrated sulfuric acid, are measured 2, 3, or 5 cc. of the standard phosphate solution for urine, the amount being determined by the result found for inorganic phosphate. Having been brought to the same volume with phosphate-free water, the standard solution and urine are treated and compared as in the determination of inorganic phosphate. If the urine is compared with the standard set at 20 mm., the calculation is as follows:

$$\frac{2 \times \text{cc. standard used}}{\text{Reading}} = \text{Gm. total P per liter}$$

Organic Phosphorus.—As pointed out by Taylor and Miller (1914), it is not permissible to estimate organic phosphorus by subtracting inorganic from total phosphorus since the amount of organic phosphorus is so small. They recommend precipitation of the inorganic phosphate with barium and determination of the organic phosphorus in the filtrate. The technique is as follows: 20 cc. of the urine are measured into a 25 cc. volumetric flask, made just alkaline with powdered barium hydroxide, filled to the mark with water, and filtered. The precipitate contains barium sulfate and phosphate. To remove the excess of barium, 20 cc. of this filtrate are measured into another 25 cc. flask, made just acid with dilute sulfuric acid, filled to volume, and filtered. An excess of sulfuric acid is to be avoided since it affects the final acidity of the determination. 1 cc. of this second filtrate is equivalent to 0.64 cc. of the original urine.

---

[2] We have not found that the addition of cane sugar as recommended by Bloor has any influence on the amount of phosphorus obtained by our method. It is possible that phosphoric acid compounds of the type mentioned by Bloor might give the full amount of color by our method and yet fail to be precipitated by his strychnine molybdate reagent. On the other hand, pyro- and *m*-phosphoric acids do not form phosphomolybdic acid and so escape determination by our method.

We have made a few determinations of organic phosphorus in the following manner: 10 cc. (equivalent to 6.4 cc. of urine) of filtrate prepared as above are evaporated with 8 to 10 drops of concentrated sulfuric acid to about 2 cc., and 2 cc. of concentrated nitric acid are added. The digestion is carried out as described under "Total phosphorus" and the residue transferred to a 25 cc. flask with 10 cc. of distilled water. In another similar flask are placed 5 cc. of the dilute standard solution for blood (equivalent to 0.025 mg. of P), 4 to 6 drops of concentrated sulfuric acid, and 5 cc. of water. To both flasks are added 1 cc. of the molybdic acid solution and 2 cc. of the hydroquinone. After 5 minutes, 10 cc. of the carbonate-sulfite solution are added, and the flasks made up to volume and read as usual, except that it is better to set the standard at 30 or 40 mm. instead of 20 mm. If the urine is read against the standard set at 40 mm., the calculation is as follows:

$$\frac{1,000}{\text{Reading} \times 6.4} = \text{Mg. organic P per liter}$$

Urines high in organic phosphorus may be read against a stronger standard. It is better, for most urines, to use the dilute standard described above, rather than to increase the amount of urine filtrate used, since the smaller the amount of urine, the easier is the digestion.

Determinations of organic phosphorus should be done on fresh urines, since, as pointed out by Mathison (1909), the values decrease on standing.

We have also determined the organic phosphorus in urine by precipitating the inorganic phosphate with magnesium citrate as described below, and estimating the phosphorus in the filtrate. The values thus obtained are slightly higher and probably more accurate than those obtained by the barium precipitation, but the determination is not so convenient and requires at least 8 hours for complete precipitation.

Accuracy.—We attempted to check the results secured on urines by these methods against gravimetric determinations of inorganic phosphate. 50 cc. portions of urine were precipitated with magnesium citrate according to the technique of Mathison (1909) and filtered after standing over night. The paper and precipitate were dried over night at room temperature and weighed. The precipitate was then brushed off the paper and the paper reweighed as suggested by Jones (1916).[3]

Table I gives the values for inorganic phosphate obtained by this gravimetric process, by our colorimetric method, and by the uranium titration, using ferrocyanide as indicator. The results by the uranium titration are usually distinctly higher than by the other two methods. The colorimetric and gravimetric methods both give good duplicate determinations, but the colorimetric values are higher.

That the difference between the results by the gravimetric and colorimetric methods is due to too low results by the former, is indicated by the values in Table II. The determinations in this series were done just as in the first series except that the filter papers, after reweighing, were extracted with dilute sulfuric acid and the phosphate thus recovered was determined colorimetrically. The first column under gravimetric determination in Table II gives the result calculated from the weight of precipitate obtained, the second column gives the additional amount calculated from the precipitate adhering to the filter paper, and the third column gives the sum of these two. In this second series, the results by the colorimetric and the corrected gravimetric methods are in much better agreement than in the first series. The results by the uranium titration tend to be higher than those by the other methods, and this is especially true in the urines containing albumin and bile.

Although the filter paper (7 cm., Schleicher and Schull's No. 589, black ribbon) was as small as could be conveniently used, examination of the column of corrections shows that quite large

---

[3] This method of determination was suggested to us by Dr. C. H. Fiske of this department, to whom we are also indebted for other valuable suggestions.

TABLE I.

Inorganic Phosphate as P per Liter.

Urine.	Gravimetric.	Colorimetric.	Uranium.
	gm.	gm.	gm.
1. Normal mixed.		0.135	0.145
		0.135	
2. " "		0.119	0.127
		0.119	
3. " "	0.5568	0.555	0.562
	0.5564	0.553	
4. " "	0.2388	0.253	0.262
	0.2384	0.255	0.260
5. " "	0.1356	0.143	0.145
	0.1356	0.142	0.146
6. " "	0.2899	0.294	0.310
	0.2899	0.294	0.307
7. Sugar 1 per cent; $FeCl_3$+++.	0.3954	0.421	0.415
	0.3954	0.416	0.414
8. " 2 " " $FeCl_3$+++.	0.1911	0.216	0.213
	0.1915	0.218	0.210
9. No sugar; $FeCl_3$+++.	0.3706	0.406	0.392
	0.3713		0.389
10. Albumin +++.	0.4408	0.455	0.445
	0.4417		0.450
11. Normal mixed.	0.3000	0.308	0.313
	0.3012		
4a. "	0.2850	0.284	
8a. Sugar present.	0.1918	0.211	0.209

TABLE II.

Inorganic Phosphate as P per Liter.

Urine.	Gravimetric.			Colorimetric.	Uranium.
	Weighed.	Correction.	Corrected value.		
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
12. Normal mixed.	0.5739	0.0028	0.5767	0.584	0.564
	0.5693	0.0073	0.5766		0.570
	0.5703	0.0056	0.5759		
13. Sugar 2.3 per cent; $FeCl_3$++; albumin +.	0.2315	0.0090	0.2405	0.235*	0.263
					0.269
14. Sugar 0.1 per cent; $FeCl_3$–.	0.2169	0.0068	0.2237	0.227	0.225
	0.2134	0.0096	0.2230		0.224
14a. Same as No. 14, 2 days later.	0.2399	0.0084	0.2483	0.244	
15. Sugar 0.2 per cent; $FeCl_3$+++.	0.1244	0.0010	0.1254	0.124	0.127
	0.1254	0.0007	0.1261		
16. Sugar 1.5 per cent; $FeCl_3$++.	0.1840	0.0048	0.1888	0.188	0.193
	0.1858	0.0035	0.1893		0.195
17. Sugar 0.4 per cent; $FeCl_3$–; bile ++.	0.4649	0.0192†	0.4841	0.485	0.515
	0.4700	0.0167†	0.4867		
18. Normal mixed.	0.2599	0.0035	0.2634	0.258	0.269
	0.2599	0.0035	0.2634	0.260	0.271
19. " "	0.1524	0.0030	0.1554	0.157	0.162
	0.1532	0.0025	0.1557	0.158	0.162
20. " "	0.1636	0.0018	0.1654	0.166	0.169
	0.1641	0.0015	0.1656	0.167	0.170

* This value was obtained after clearing with carbon. The urine contained much mucus and was very turbid.

† The precipitate in this urine was extremely fine so that some of it passed through the filter. This was collected by centrifuging, and is included in the correction.

and variable amounts of precipitate adhere so firmly that they cannot be brushed loose. The amount of this adhering precipitate varies with different urines, though it is usually nearly the same in duplicate determinations, and it is perhaps dependent on variations in crystal size and shape in different urines. The urines having the largest corrections are those containing albumin and bile. When dealing with 50 cc. of urine, it is evident that satisfactory duplicate determinations by weighing magnesium ammonium phosphate are no guarantee of accuracy. However, the variations obtained with different urines and the low correction in certain urines (Nos. 15 and 20, Table II) suggest that with other material the adhering precipitate may be much less.

Blood.

For the determination of the inorganic and acid-soluble phosphorus in blood we have made use of trichloroacetic acid. The acid ammonium sulfate used by Bloor (1918) cannot be conveniently used with our method on account of the large amount of salts in the filtrate. The tungstic acid filtrates of Folin and Wu (1919) give consistently low results, probably due to the formation of phosphotungstic acid from the tungstic acid reagent and the inorganic phosphate of the blood and combination of this substance with the protein precipitate. The acetic-picric acid filtrates of Greenwald (1916) cannot be used for the determination of inorganic phosphate by our method on account of the interference of the color of the picric acid. Acetic-picric acid filtrates can be used for the determination of the total acid-soluble phosphorus if the picric acid is driven off as described by Greenwald and the residue digested with nitric and sulfuric acids. We have always found trichloroacetic acid to be free from phosphorus, but all samples should be tested.

We have found that a dilution of 1:5 is sufficient for normal plasma and gives the same result as a dilution of 1:10. If the plasma is suspected to be high, or if whole blood is used, we recommend a dilution of 1:10. 5 cc. of plasma are mixed with 15 cc. of distilled water in a 25 cc. volumetric flask and 5 cc. of 20 per cent trichloroacetic acid added with shaking. The flask is filled to the mark with water, mixed, and after 10 minutes is filtered through a phosphate-free (acid-washed) filter. If whole blood is used, 5 cc. are laked by mixing with 35 to 40 cc. of distilled water in a 50 cc. volumetric flask and 5 cc. of 20 per cent trichloroacetic acid are added with shaking. The flask is made up to volume, and filtered after 10 minutes.

Inorganic Phosphate.—To 10 cc. of the trichloroacetic acid filtrate in a 25 cc. volumetric flask are added 1 cc. of the molybdic acid solution and 2 cc. of the hydroquinone solution. At the same time, molybdic acid and hydroquinone are added to a similar flask containing 10 cc. (equivalent to 0.05 mg. of P) of the dilute standard phosphate solution for blood, and either 1 or 2 cc. of 20 per cent trichloroacetic acid, according to whether a dilution of 1:10 or 1:5 has been used. After 5 minutes, 10 cc. of the carbonate-sulfite solution are added, and the flasks are made up to volume and mixed. The colors are compared after 5 to 10 minutes. If the unknown is read against the standard set at 20 mm., the calculation is

$$\frac{100}{\text{Reading}} = \text{Mg. P per 100 cc. (if 1:10 dilution was used)}$$

or

$$\frac{50}{\text{Reading}} = \text{Mg. P per 100 cc. (if 1:5 dilution was used)}$$

It may be necessary to use a stronger standard or less filtrate in bloods containing much phosphorus. The proper standard and amount of filtrate may be roughly estimated before the determination is made. A 5 cc. mark is made on two small similar test-tubes. In one of these tubes is placed 1 cc. of the dilute standard phosphate solution for blood and in the other, 1 cc. of filtrate. About 10 drops of molybdic acid and hydroquinone solutions are added to both tubes, and after a few minutes the tubes are filled to the mark with carbonate-sulfite solution. The amount of color may be roughly gauged and the strength of standard and amount of filtrate decided upon. Serum or plasma may be roughly estimated in the same manner before removing the proteins in order to judge the proper dilution to use.

The inorganic phosphate must be determined in the trichloroacetic acid filtrate as soon as possible after filtering since the acid hydrolyzes the organic phosphorus on standing, giving too high results. After standing 24 hours, the values for inorganic phosphate and total acid-soluble phosphorus are nearly identical. One plasma, containing 9.6 mg. of acid-soluble phosphorus per 100 cc., gave the following values for inorganic phosphate: 30 minutes after filtering, 8.8 mg.; 18 hours after filtering, 9.4 mg. Another plasma, containing 3.5 mg. of acid-soluble phosphorus, gave the following values for inorganic phosphate: 30 minutes after filtering, 3.2 mg.; 90 minutes after filtering, 3.4 mg.; 5 hours after filtering, 3.4 mg.

TABLE III.

Inorganic and Total Acid-Soluble Phosphorus as P per 100 Cc.

Specimen.	Inorganic phosphate.	Acid-soluble phosphorus.	
		Trichloroacetic acid.	Acetic-picric acid.
	mg.	*mg.*	*mg.*
1. Plasma, human	3.5	5.0	
2. “ “	3.9*	4.0	
3. Whole blood, beef	4.9	8.3	
	5.1	8.6	
4. “ “ “	5.9	11.6	
5. Plasma, human (hemolysis)	9.0	10.5	
6. “ “ (diabetic)	4.4	5.1	
7. “ “ “	4.4*	4.4	
8. “ “ “	4.5	5.0	
9. “ “ “	4.8†	4.7	4.8
10. “ “ (hemolysis)	8.8	9.8	9.6
11. Whole blood, beef	5.1	10.3	10.3
12. Plasma, human (diabetic)	3.2	3.6	3.5
13. “ beef	8.0*	8.1	8.0
14. Same as No. 13 with added phosphate (40 mg. per 100 cc.)	47.7		

* These specimens were several days old when analyzed.

† The filtrate from this specimen stood 24 hours before the determination was carried out.

Total Acid-Soluble Phosphorus.—The total acid-soluble phosphorus is determined in a manner similar to the total phosphorus in urine. 10 cc. of the trichloroacetic acid filtrate, in a hard glass test-tube, are evaporated down to about 2 cc. with 6 to 8 drops of concentrated sulfuric acid and a piece of quartz. 1 cc.

of concentrated nitric acid is added and the digestion continued until all the nitric acid has been driven off and the remaining drops of sulfuric acid are clear. The residue is transferred to a 25 cc. volumetric flask with about 10 cc. of distilled water and treated as under inorganic phosphate. The standard to be used is determined by the value previously found for inorganic phosphate. 4 to 6 drops of concentrated sulfuric acid are added to the standard to balance that used in the digestion but no trichloroacetic acid is added, since all of this substance is volatilized during the digestion.

Results.—Table III gives some values obtained by these methods. The agreement between the trichloroacetic acid and picric acid filtrates is satisfactory. The ratio of inorganic to total acid-soluble phosphorus is about that usually found by other methods.

SUMMARY.

Rapid colorimetric methods are described for the determination of inorganic, organic, and total phosphorus in urine. While not so accurate as suitable gravimetric determinations, these methods are much more convenient and are sufficiently accurate for many purposes. The method for inorganic phosphate appears to be more accurate and rapid than the usual uranium titration.

Methods are also described for the determination of inorganic and acid-soluble phosphorus in blood.

BIBLIOGRAPHY.

Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.
Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.
Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
Greenwald, I., *J. Biol. Chem.*, 1916, xxv, 431.
Jones, W., *J. Biol. Chem.*, 1916, xxv, 87.
Mathison, G. C., *Biochem. J.*, 1909, iv, 233.
Taylor, A. E., and Miller, C. W., *J. Biol. Chem.*, 1914, xviii, 215.

THE EFFECT OF DIET ON THE EXCRETION OF INDICAN AND THE PHENOLS.*

By FRANK P. UNDERHILL and GEORGE ERIC SIMPSON.

(From the Department of Experimental Medicine, School of Medicine, Yale University, New Haven.)

(Received for publication, July 13, 1920.)

Since Baumann's epoch-making paper on urinary phenol, indican, and the ethereal sulfates (1), considerable work has been done on the quantitative interrelations of these substances, and on the effect of diet on their excretion. Results have been obtained as contradictory as the number of the investigators so that, in these aspects, the literature since Baumann is a dreary desert of mirages. And the reason is not hard to find. In the first place, Folin and Denis (2) have shown that ethereal sulfates must necessarily represent a varying amount of phenol, since the degree of conjugation of these substances is at times quite variable. Furthermore, the methods for phenol determination used by the earlier workers were so crude that only a small part of the urinary phenols could have been accounted for. The phenols, after concentration in alkaline solution and distillation from acid solution, were determined either gravimetrically (3), by precipitation with bromine; or volumetrically (4), by titration with iodine. The latest modification of these methods was proposed in 1912 (5). It still involved prolonged heating with alkali which destroys phenols, as Folin has shown. It is evident that clearer knowledge had to wait on better analytical methods and that, the method at hand, the time is ripe for a study of the effect of diet on the excretion of these substances.

The pioneer work of Herter and Kendall (6) and, more recently, of Rettger and his coworkers (7–9), and of Torrey (10, 11) shows that diet is effective in regulating the character of the intestinal flora. It is, therefore, of prime importance to know the effect of diet on the products of bacterial metabolism eliminated in the urine. Are the changes which are effected in bacterial flora reflected in the quantitative excretion of these products of their metabolism? The most important of these are the aromatic hydroxyacids or phenols, and indican. By putting a patient on a certain regulated diet, can it be said that the processes of bacterial metabolism have thereby been automatically affected, or is it necessary in order to ascertain the efficacy of certain food-stuffs in the regulation of intestinal putrefaction that the bacterial content of the feces be determined? Would the determination of the phenol and indican excretion give the same information?

---

* The data in this paper are taken from the dissertation presented by George Eric Simpson for the degree of Doctor of Philosophy, Yale University, 1920.

The present paper gives the results of a quantitative study of the effects of diet on the phenol and indican excretion of the normal individual. By comparison of these results with those of Herter, of Rettger, and of Torrey, we should be able to ascertain how closely excretion of these substances is related to the extent of putrefactive processes in the intestine.

HISTORICAL.

Herter and Kendall (6) determined the effect of diet on the processes of intestinal putrefaction by qualitative bacterial analysis of the fecal flora. When the subjects of their experiments, kittens and monkeys, were on a diet of milk and cane-sugar, the acidophiles rapidly replaced the putrefactive microorganisms which predominated when eggs or meat were the chief constituents of the dietary. They attributed the change to the dominant position of carbohydrate in the milk and cane-sugar diet.

Our present day interest in the effect of diet on the bacteria of the intestinal tract has been crystallized by the work of Rettger and his pupils who have laid especial stress on the influence of lactose in changing the flora in the intestine of rats or of man from predominantly putrefactive to predominantly acidophilic in character. Torrey (10), working on dogs, found that lactose and dextrin were both highly efficacious in transforming the flora of these animals. As little as 30 gm. of lactose or dextrin added to a diet of rice and beef heart were able to produce this change. When starchy foods were fed, there was always a tendency toward the simplification of intestinal flora with elimination of the putrefactive types.

Hirschler (12), a pupil of Baumann's, was the first to demonstrate by *in vitro* experiments the inhibitory action of carbohydrates on intestinal putrefaction. Underhill (13), in feeding experiments with dogs, showed that indican excretion was related to the tryptophane building-stone of the ingested protein. Homer (14), on the other hand, was unable to find any increased excretion of indican after feeding tryptophane and believed that bacterial action on tryptophane was not a factor in indican formation.

After the development of an accurate method for phenols, Folin and Denis (15–17) were able to carry out far more phenol determinations than had ever previously been reported, and thus provided our first accurate knowledge of the quantitative elimination of these substances. This knowledge of the phenol excretion has been extended by the work of Dubin (18, 19). Moore (20) applied the method to a study ofphenol elimination in infants and found that phenol excretion varied directly with the protein intake, in agreement with Folin's results on the adult.

EXPERIMENTAL.

Since the development of Folin's method for phenols in urine, an extended study of phenol and indican excretion has not been carried out on the normal individual. Such a study is necessary in order that an intelligent interpretation of pathological data may be possible. It has been shown by the results of previous workers that such a study to be of value should meet certain requirements. Any diet should be given for a sufficient time that its effect on the bacterial flora and hence, presumably, on indican and phenol excretion, may become completely established. Since Dubin has shown in his work on dogs that given a uniform diet the phenol excretion tends to remain almost constant in the individual, while the level of elimination may vary markedly in *different* individuals, the effect of various diets should be determined in a single individual in order that comparison may be possible.

For the duration of the experiment artificial stimulation of peristalsis should be excluded, since it would introduce a variable which has been shown to lower the urinary phenol excretion considerably. Furthermore, natural evacuation of the bowel should be regular and complete.

General Plan of the Investigation.

The present paper is based on studies in phenol and indican excretion of a dog extending over a period of 3 months, and of a normal student for a consecutive period of 1½ months at one time and for 15 days at another time. The excretion by two other normal males was followed for shorter periods. The effect of the following nitrogenous foods, when ingested in large amount, was investigated: gelatin, a tryptophane-free source of nitrogen; meat, a typical animal protein; liver, as an example of glandular tissue high in nuclear material; kidney beans, a vegetable protein presented in the form in which such proteins are usually found in the dietary, that is mixed with a certain amount of indigestible carbohydrate material; and glidine, a mixture of wheat proteins freed from carbohydrate material.

The effect of the carbohydrates, lactose, dextrin (contained in Zwiebach), and starch, was studied in three different individuals. These experiments are not so valuable as they would have been had it been possible to carry them out entirely on the same individual. Nevertheless, they are extremely suggestive.

Methods.

During the course of these experiments, 24 hour urine samples were collected. Since it has been shown by Folin and Denis (17) and by Dubin that little phenol is excreted in the feces compared to the amount in the urine; and by Bergeim (21) that the amount of indole in the feces is exceedingly small, the feces were not collected. On the urines, the determination of total nitrogen, creatinine, phenols, free and combined, and indican was carried out. Total nitrogen was done by the macro Kjeldahl method, creatinine by the method of Folin, indican by Ellinger's permanganate titration method (22), and phenols by the method of Folin and Denis.

All determinations were carried out in duplicate, except the creatinine. When there was a tendency toward variation in the amount of creatinine, duplicate determinations were performed here also.

In the experiments on man it was not found feasible or believed advantageous to regulate the diet completely. At the beginning of the period for urine collection, the experimental diet was taken. This was at noon. For this meal, two rolls were eaten with the experimental nitrogenous mixture. It was found necessary to heat the gelatin and casein with substances to modify their taste. Half the gelatin was mixed with some canned chicken soup and eaten hot. The rest was mixed with a teaspoonful of sugar and the juice of a lemon and frozen hard. The casein was boiled for some time with stewed tomatoes. A homogeneous, not unpleasant tasting mixture resulted The meat was freed from visible fat, ground, and boiled for ½ hour with a small amount of water. The fat which separated out was poured off, as it was found that otherwise digestive distress ensued. The liver was weighed out and fried. The larger pieces of connective tissue were not eaten. The lactose was mixed with a cup of water and a drop of vanilla extract and taken undissolved.

For supper, Individual A received a small amount of meat (less than ⅛ pound), some potatoes, bread or other food high in carbohydrate, and fruits or vegetables of large cellulose content. For breakfast a roll, prunes, and milk were taken. An endeavor was made to keep the amount of roughage at all times approximately constant. A record was kept of the diet to aid in the interpretation of possible anomalies. During the protein periods the experimental constituents of the diet far overshadowed in quantity, and it is believed in effect on intestinal putrefaction, any other constituents of the diet.

In the experiments on high carbohydrate feeding, carried out on Individuals A, B, and C, the diet contained no meat or legumes. Individual A took 100 gm. of lactose two to four times a day; Individual C, 1¼ pounds of Zwieback to provide a "practical" dextrin diet. The basis of Individual B's diet was rice, bread, potatoes, bananas, and other foods containing a large amount of starch. Individuals A and C supplemented their experimental diet with foods of high starch content.

During the low nitrogen periods, the dog received the following diet:

3.4	gm.	nitrogen as meat, gelatin, casein, kidney beans, etc.
1.1	"	" contained in meat.
110	"	cracker meal containing 2.1 gm. nitrogen.
26	"	lard.
2	"	bone ash.

On the higher level of nitrogen intake, the amount of cracker meal was reduced by half—from 110 to 55 gm.—and the protein intake increased two- to threefold to give the calorific equivalent of the cracker meal.

In the experiments in which 100 gm. of lactose were fed daily in addition to a large amount of meat, the lard was omitted. The diet consisted of:

417	gm.	meat.
100	"	lactose.
55	"	cracker meal.
2	"	bone ash.

Lest the presence of fat might be a factor in phenol and indican excretion, during the meat period which followed these lactose-meat periods, it was also omitted, since it was desired to compare the effect of the two diets (see Table IV, D89, 90, and 91).

From the meat most of the fat and connective tissue was removed. It was ground up and preserved on ice in small sterile jars until needed. The beans were prepared by boiling in an autoclave at 15 pounds pressure for 4 hours. The resulting mush was analyzed for nitrogen and substitution was made accordingly. No account was taken of the appreciable proportion of nitrogen which here did not represent protein. The casein was a preparation made 2 or 3 years ago. Cooper's gelatin was used. Merck's milk sugar was used in the lactose experiments. A mixture of wheat proteins was supplied by glidine, a proprietary preparation put up by Menley and James, New York.

Experiments on Man.

Dubin, in his work on the dog, found that when the animal had been on an unvarying diet for a few days the excretion of phenols showed a high degree of constancy, the extreme variations falling under 10 mg. a day. In our dog experiments, phenol excretion attained the same degree of constancy in those cases where the animal was kept for several days on a diet in which meat, casein, or beans were fed (Table III, D1–41).

Our experiments on the human being have not shown this constancy to be characteristic of the subjects of these experiments. Our diets were not so strictly regulated as those of the dogs in Dubin's experiments, especially as to water intake, since it was not found feasible to regulate this carefully.

Then too, the effect of diuresis explains many of the variations in phenol excretion, especially those unexpected high values obtained sometimes, which are not related to diet changes. Thus, Individual C (Table II, C5–8), while on a high carbohydrate diet, had been excreting an amount of phenol within the range of excretion of the other subjects on similar diets. However, on January 6, the excretion markedly increased—from 295 to 522 mg. of phenol in 24 hours. This increase is associated with, and is to be explained by, the sharp increase in the volume of the urinary output. Thus, in the days when the urine volume was low (C2–4), an average of 740 cc. being excreted daily, the phenol excretion was also low, averaging 342 mg. daily, as compared with that of the 3 days when the urine volume averaged 1,158 cc. and the phenol excretion increased to an average of 530 mg. daily (C5–7).

There is still another factor. In man, certain inhibitions may interfere with the time, and hence probably with the completeness of the evacuation of the bowel, also. In the case of the dog, however, this is not a factor.

That the effect of change of diet on the formation of phenols is completely overshadowed by the factor of bowel evacuation is shown by the high phenol values when the regularity of defecation is interfered with. Although on the previous day there had been a sweeping out of phenol, associated with diuresis, Subject A, on August 5 (Table I, A8), eliminated a much larger amount of phenol, due in part to diuresis, in part, undoubtedly, to failure in evacuation of the bowel. This effect is also seen on August 15, when defecation occurred three times within 24 hours and phenol excretion decreased. But the effect is seen most clearly when the subject was on a meat diet and frequency of defecation increased. The decreased phenol and indican excretion on August 24 and on September 15 is due to no other cause. Thus in days A27 and A39, there was a decrease of over 100 mg. in the amount of phenol eliminated that is not associated either with diuresis or diet changes. On January 1 the effect is even more marked, where, as the result of enhanced evacuation, the urinary excretion of phenol and indican both fell, phenol from 410 to 147 mg. and indican from 23 to 15 mg.

TABLE I.

Indican and Phenol Excretion in Man: Nitrogenous Food-Stuffs.

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P:N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
A1	July 29	Unregulated diet;	795	10.7	1.11	425	216	209	51	49	3.9	3.9
A2	" 30	preliminary.	1,145	12.7	1.27	376	240	136	64	36	3.0	11.0
A3	" 31		1,490	10.3	1.47	506	314	192	62	38	5.1	12.7
A4	Aug. 1		1,200	12.8	1.32	440	231	209	53	47	3.4	10.8
A5	" 2	50 gm. gelatin.	1,110	9.8	1.37	432	262	170	60	40	4.4	6.9
A6	" 3	100 " "	1,670	19.7	1.54	461	278	183	60	40	4.8	10.4
A7	" 4		2,080	20.2	1.54	555	256	299	46	54	2.8	Trace.
A8	" 5	0*	1,890	21.6	1.57	600	331	269	55	45	2.5	"
A9	" 6		2,165	22.3	1.34	525	300	225	57	43	2.3	"
A10	" 7	100 gm. casein.	1,700	20.3	1.56	676	435	241	64	36	3.3	"
A11	" 8		1,510	19.0	1.51	527	349	178	66	34	2.7	11.0
A12	" 9		1,073	19.1	1.47	429	248	181	58	42	2.2	7.3
A13	" 10	100 gm. gelatin. 0*	995	17.8	1.55	478	276	202	58	42	2.7	8.5
A14	" 11		1,300	18.4	1.47	349	214	180	49	51	1.9	Trace.
A15	" 12		1,073	22.9	1.54	471	245	226	52	48	2.2	"
A16	" 13		1,580	19.4	1.10	527	264	263	50	50	2.7	"
A17	" 14		2,060	21.8	1.48	548	278	270	51	49	2.5	"

---

* The number indicates defecations when this was different from one daily.

A18	Aug. 15	100 gm. casein. 3*	1,415	19.1	1.52	498	259	239	52	48	2.6	1.6
A19	" 16	0*	1,520	18.8	1.38	437	254	183	58	42	2.3	5.6
A20	" 17		1,000	17.3	1.39	417	227	190	54	46	2.4	9.6
A21	" 18		1,420	19.9	1.42	455	257	198	57	43	2.3	10.5
A22	" 19	500 gm. meat.	1,715	19.1	1.57	513	274	239	53	47	2.7	20.4
A23	" 20		1,908	13.4	1.73	525	308	217	59	41	3.9	11.9
A24	" 21	Epistaxis.	1,308	20.8	1.52	661	473	188	72	28	3.2	8.2
A25	" 22	"	1,470	22.2	1.60	521	286	235	55	45	2.3	21.7
A26	" 23	" 4*	1,210	20.4	1.73	466	240	220	52	48	2.3	20.5
A27	" 24	" 2*	920	14.1	1.58	348	179	169	51	49	2.5	6.7
A28	" 25	Influenza.	1,307	11.2	1.79	388	208	180	54	46	3.5	20.0
A29	Sept. 5	100 gm. casein, 100	1,140	17.8	1.52	570	257	313	45	55	3.2	5.8
A30	" 6	gm. lactose.	1,240	22.4	1.70	434	261	173	60	40	1.9	9.2
A31	" 7		966	19.3	2.24	532	260	272	49	51	2.8	11.4
A32	" 8	100 gm. casein.	860	17.0	1.51	430	220	210	51	49	2.5	7.8
A33	" 9		1,155	21.2	1.58	421	226	195	54	46	2.0	6.5
A34	" 10		1,410	18.4	1.65	473	263	210	56	44	2.6	9.2
A35	" 11		1,450	21.0	1.59	473	321	152	68	32	2.3	4.5
A36	" 12	500 gm. meat, 100	1,555	21.7	1.71	430	238	192	55	45	2.0	11.5
A37	" 13	gm. lactose.	1,220	18.7	1.72	406	218	188	54	46	2.2	15.9
A38	" 14		1,000	22.0	1.70	486	245	241	51	49	2.2	15.9
A39	" 15	2*	1,250	21.6	1.70	380	224	156	59	41	1.8	19.0

TABLE I—*Concluded.*

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P: N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		
A40	Sept. 16	500 gm. meat.	1,773	22.5	1.84	531	297	234	56	44	2.4	10.1
A41	" 17	Epistaxis.	1,400	19.7	1.71	432	243	189	56	44	2.2	10.7
A42	" 18	"	1,520	21.7	1.90	447	244	203	55	45	2.1	19.6
A43	" 19	"	1,170	20.5	1.77	421	243	178	58	42	2.0	13.3
A44	Dec. 29	Regular diet.	1,270	10.3	1.87	278	269	9	97	3	2.7	18.0
A45	" 30		957	10.0	1.84	172	148	24	86	14	1.7	17.0
A46	" 31	500 gm. liver.	1,650	18.3	2.03	410	315	95	77	23	2.2	23.0
	1920											
A47	Jan. 1	2*	835	18.5	1.70	147	109	38	74	26	0.8	15.0
A48	" 2		1,040	17.5	1.81	295	214	81	73	27	1.7	20.0

Effect of Diet on Phenol and Indican Excretion.

General.—In the periods when proteins were a preponderating constituent of the diet of Subject A (A1–43) the elimination of phenol is well over 400 mg. a day. The highest value for the daily excretion of total phenols, obtained when defecation frequency was not an interfering factor, was 676 mg. (A10), the lowest 349 mg. (A14), the average, 476 mg.

In the low protein periods, where large amounts of lactose were ingested, however, the phenol excretion was always well under 400 mg. An average of 311 mg. a day was excreted during the 8 days of this régime. Curiously enough, this decrease in phenol excretion on the low protein, high lactose diet is not associated with a decrease in indican excretion. In fact, when we come to consider the experiments on Subjects B and C as well as A, it is seen that indican and phenol excretion may vary quite independently of one another. Thus, Individual B, on a diet containing much carbohydrate, excreted much more indican than Individual A, even on a meat diet, although this diet gave a much higher indican excretion than the lactose diet, if we compare the results on Individual A *inter se.* Nevertheless, the phenol excretion of Individuals B and of A on the carbohydrate diets was of the same degree. Quite different is the case with Subject C. In only one instance did this individual excrete detectable amounts of indican. This individual was excreting far more phenols than either Subjects B or A, although all were on a diet containing large amounts of carbohydrate. Phenol and indican excretion, then, in different individuals, may bear quite a different relation to one another. May this be associated with differences in the character of the flora—certain strains being especially prone to metabolize tryptophane, for instance?

Consideration of the ratio of the total phenols to total nitrogen should answer the question: Does total phenol excretion parallel the total nitrogen of the food? When there is incomplete absorption of the food, shown by a low nitrogen excretion, is there a tendency toward the manufacture of indican and phenol in larger amounts than usual? In the experiments on man it is seen that this ratio tends to become constant when the diet is regulated, and that the highest values are found during the high

TABLE II.

Indican and Phenol Excretion in Man: Carbohydrate Diet.

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P:N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
Subject A: Lactose.												
	1920		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
A49	Jan. 3	200 gm. lactose.	990	9.1	2.10	249	200	49	80	20	2.7	10
A50	" 4	200 " "	980	6.9	2.02	297	175	122	59	41	4.3	12
A51	" 5	400 " "*	1,370	6.4	2.04	311	182	129	58	42	4.9	8
A52	" 6		1,440	5.8	1.96	327	185	142	56	44	5.7	6
A53	" 7		1,055	5.3	1.99	377	203	174	54	46	7.1	11
A54	" 8		1,200	5.6	1.78	314	177	137	56	44	5.6	4
A55	" 9											
A56	" 10		580	4.5	1.77	268	132	136	49	51	5.9	4
A57	" 11		1,335	6.4	1.94	338	236	102	70	30	5.3	9
A58	" 12		760	5.9	1.52	260	170	90	65	35	4.4	11
Subject B: Starch.												
B1	Jan. 2	Regular diet.	850	9.1	1.98	424	228	196	54	46	4.7	7
B2	" 3	Starch "*	1,590	10.6	2.02	310	239	71	77	23	2.9	27
B3	" 4		1,120	8.9	1.81	307	193	114	63	37	3.5	34
B4	" 5		1,325	10.3	1.92	306	195	111	64	36	3.0	32
B5	" 6		1,030	8.8	1.70	262	153	109	58	42	3.0	23
B6	" 7		830	9.8	1.77	392	184	208	47	53	4.0	30

* This diet continued for the duration of the experiment.

B7	Jan. 8		1,140	10.9	1.73	373	185	188	50	50	3.4	17
B8	" 9		870	10.3	1.83	353	203	150	58	42	3.4	34
B9	" 10		1,520		2.02	387	225	162	58	42		23
B10	" 11		1,080	9.5	1.97	378	241	137	64	36	3.9	14
B11	" 12		1,145	9.2	1.63	394	263	131	67	33	4.3	10
B12	" 13		660	9.6	1.46	389	258	131	66	34	4.0	17
Subject C: Dextrin.												
C1	Jan. 2	Regular diet.	825	8.2	1.90	393	169	224	43	57	4.8	None.
C2	" 3	Dextrin "†	880	8.2	1.93	383	245	138	64	36	4.7	"
C3	" 4		690	9.3	2.70	349	216	133	62	38	3.7	"
C4	" 5		650	9.9	2.20	295	116	179	39	61	3.0	"
C5	" 6		1,350	9.1	1.67	522	318	204	61	39	5.7	"
C6	" 7		1,230	8.2	1.84	550	301	249	55	45	6.6	"
C7	" 8		895	11.7	1.79	516	162	344	33	67	4.4	"
C8	" 9											
C9	" 10		890	11.7	1.72	450	247	203	55	45	3.8	None.
C10	" 11											
C11	" 12		1,130	7.4	2.08	464	354	110	76	24	6.2	16.0
C12	" 13		900	8.0	1.66	417	292	125	70	30	5.2	None.

† The diet contained approximately 1½ pounds of Zwiebach for the rest of the experimental period.

lactose period. Is this, perhaps, due to the stimulating effect of this relatively insoluble carbohydrate on bacterial metabolism so that, from the small amount of nitrogen available, a comparatively large amount is utilized by the microorganisms? It cannot be overlooked that in this case the small amount of protein was supplied by potatoes and bread. The coefficient of digestibility of these proteins has been shown by Mendel and Fine (23) to be the same as that of meat. In the form of bread, however, the protein is evidently protected from the action of the digestive juices since the nitrogen is less available than that of meat, as shown by Woods and Merrill (24). In this case, therefore, more undigested residue may reach the sphere of action of the bacteria in spite of the low nitrogen intake, and thereby give rise to an increased excretion of indican and phenol. Of the two influences, it cannot be determined which was effective.

In the period during which the higher level of nitrogen intake was maintained, the conjugated phenols represented between 40 and 50 per cent of the total phenols. In only four instances did the degree of conjugation fall below this limit (Table I, A10, 11, 24, and 35). In the first and third, the decrease was associated with a sudden increase in total phenols, with the actual amount of conjugated phenols remaining at the same level. In general, it may be said that the degree of conjugation remained the same in spite of the wide differences in nitrogen intake. The extent of conjugation rose and fell with the total phenol excretion except when sudden variations were encountered. The stimulating effect of high phenol excretion on the degree of conjugation, noted by Dubin, is not apparent from our experiments, except that the actual amount of conjugated phenols increased enough to keep the proportion of conjugated phenols the same.

Gelatin.—When gelatin was the chief source of nitrogenous food (Table I, A5–9, 13–17), there was always, within a day or two, a fall to a mere trace of indican in the urine. This reappeared as soon as the gelatin was replaced by casein. The disappearance in this case is not associated with any marked change in the phenol output. The swiftness of the disappearance and reappearance gives an idea of the time necessary for our experimental nitrogenous food-stuffs to exert their effect in predominating the metabolism of the intestinal flora. In studying the effect of casein, meat, and the meat-lactose combinations, it will be seen that phenol and indican excretion always showed a tendency to vary in the same direction in this individual. That they do not do so in this instance is merely an expression of the fact that gelatin is lacking in the building-stone which gives rise to the indican. Thus our results confirm those of Underhill, who determined the indican excretion of dogs when gelatin was fed as the predominating source of nitrogen. It was found that there was an invariable decrease on this diet, as compared with meat as the nitrogenous food.

Casein.—When casein was the chief source of nitrogen, phenol excretion was at the same level as with gelatin, while the indican excretion increased and attained a level usually somewhat under 10 mg. of indigo in 24 hours (Table I, A10–12, 18–21, 32–35). In the second period of casein feeding the casein influence was established immediately and 1.6 mg. of indigo were found in the urine collected 24 hours after its feeding had begun. This quick establishment was due to the fact that the debris from the preceding gelatin period had been removed from the sphere of action of the bacteria as the result of the increased frequency of defecation. The slight diarrhea was probably due to mechanical irritation of the walls of the intestine by particles of casein. These were sharp cornered because the casein had not been sufficiently softened by boiling before it was eaten.

Meat.—When a diet containing large quantities of meat followed a diet of casein there was always a sharp increase in the amount of indican in the urine (Table I, A22–28, 36–43). This increase appeared the 1st day the meat was fed and soon resulted in the excretion of about 20 mg. of indigo a day. During the meat period the phenol values, as well, tended to become higher than in the preceding casein régime. While this effect is not seen so clearly in the second series of meat experiments, it is invariably seen in the experiments we have carried out on the dog. During the period of meat feeding the indican values were quite variable —much more variable than on the casein diet. This diet in both cases gave rise to increased frequency of defecation. This resulted in sharp decreases in the excretion of both phenols and indican.

In both instances the meat diet gave rise to very disagreeable subjective symptoms. It caused epistaxis, as well as decreased mental and physical efficiency. During the first series of experiments on this diet, a midsummer cold developed. During the second series, symptoms of a cold were appearing, as well as the epistaxis, so that the experiment was discontinued. In view of these results the diarrhea produced on these meat diets seems to the writer to be a protective mechanism to aid in getting rid of harmful and poisonous materials.

Why should meat differ from gelatin and casein in causing the relatively high excretion of phenol and indican? There are two differences between meat and the other nitrogenous substances which may have contributed to this effect. The meat was always mixed with a certain amount of fat, which may have caused more of the protein material to pass to the lower gut undigested. The larger part of the fat was always drained off, however, and, as no butter was eaten during this period, the total amount of fat eaten was not greater than on the other diets. In the second place meat is unique among our experimental foods in having a certain mixture of indigestible protein, the connective tissue protein. Therefore, a greater amount of material containing tryptophane and tyrosine building-stones would reach the field of bacterial activity and enter into bacterial metabolism.

Liver.—The ingestion of 1 pound of liver a day (Table I, A46–48) brought about an immediate increase in the phenol and indican excretion. Although the clearness of the result was partly obscured by the very complete evacuation of the bowel on the 2nd day, it is evident that liver, rich in nuclear material, does not differ from muscle tissue in its effect on intestinal putrefaction.

Carbohydrate Foods.—The effect of the addition of small amounts of lactose to a diet high in protein was studied in order to determine if this would have an inhibitory effect on bacterial metabolism. The object of these experiments was to have present in the diet an amount of lactose which might be ingested in the form of milk. No change was seen during the few days in which this diet was fed, either in phenol or in indican excretion (Table I, A29–31, 36–39). In a course of milk feeding which lasted 5 days, 4 quarts of milk daily being ingested, no decrease in phenol or indican excretion was seen. The value for both phenol and indican was intermediate between our casein and meat values. Constipation was an interfering factor in these experiments, which could not be avoided. Therefore, these results have not been included in this report.

In a second series of experiments with carbohydrates, three subjects were kept for 8 to 10 days on a régime in which carbohydrate was supplied in much larger quantities. Rettger has shown that lactose changes the bacterial flora in the course of a few days from predominantly putrefactive to predominantly acidophilic. Torrey found that in puppies the addition of bread to a milk diet caused a considerable change in the flora of the intestine. He attributed the consequent replacement of *Bacillus coli* by *Bacillus acidophilus* to the dextrin content of the bread, in part. It seemed of interest, then, to perform experiments which should be comparative and thus determine the effect of these carbohydrates on the excretion of phenols and indican. Unfortunately, our results are not comparable *inter se* on this point since it was not possible to carry them all out on the same individual, and our original question has not been answered.

The excretion of indican and phenol by Subjects B and C did not differ materially from the excretion during the preliminary period. The results lead to one definite conclusion and that is that, by putting a subject on a diet of high carbohydrate content —a "practical" diet—whether the carbohydrate is lactose, or dextrin from toast, or starch, it cannot from that alone be said that the excretion of the products of intestinal putrefaction has ceased, or even come to a low level. In order to know what has happened as the result of such treatment it is indispensable either that studies of fecal bacteria be carried out, or that the excretion of phenol and indican be followed in a quantitative way. Thus, Subject C, on dextrin diet, on only one occasion excreted indican in the urine; Subject A, on lactose, excreted a small amount of indican; while Subject B, on starch, excreted very large amounts of indican compared with the others. The phenol excretion of all subjects was low as compared with the values obtained on Subject A on high protein diets. The high phenol values found on Subject C (C5–7) are a result of the diuresis which followed days of small urinary output.

In respect to the experiments on lactose, these may be compared with the other experiments on Subject A. On this diet the nitrogen output was low. The phenol excretion was much lower than on the high nitrogen diets. The indican also was low. The beginning of the period of lactose feeding was accompanied by the development of large amounts of gas in the alimentary tract with very marked distention of the abdominal wall. This lasted until the 7th day, when the distress was considerably diminished, while on the 8th and succeeding days there was no notable development of gas. This gas production we have interpreted as due to the predominance of the putrefactive organisms which are able to convert lactose into carbon dioxide, and the decrease in the amount of gas we have interpreted as due to the displacement of the putrefactive organisms by the acidophiles. It would have been of great interest to continue these experiments a few days longer. Unfortunately, however, it was necessary to discontinue them so that it remains for future experiments to show whether the effect which Rettger and his coworkers have found for lactose in reducing putrefactive bacteria would have been associated with a further decrease in the excretion of urinary phenols and indican.

Experiments on the Dog.

The experiments on the dog may be divided into two groups. In the first (Table III, D1-41), the nitrogen intake was comparatively low. The diet was regulated to give about 0.9 gm. of protein per kilo. The nitrogen excretion on this régime was about 5 gm. a day, except on the bean diet where absorption was impaired. In the second group of experiments the amount of carbohydrate was reduced while the protein intake was increased so that the same energy value was provided. The cracker meal allotment was reduced by half and the protein intake increased approximately threefold to make up this difference. The nitrogen excretion was twice, and sometimes threefold that on the low nitrogen diets.

For 2 weeks previous to the experimental periods, the animal had been kept on a diet of meat scraps and cracker meal. For some time after the experimental period there was a tendency for the phenol and indican values to vary considerably. The general trend was toward smaller values irrespective of the diet. Thus, during the first 34 days of the experiments the phenol excretion decreased, whether the nitrogenous food was meat (D1–8), gelatin (D9–18), casein (D19–26), or again meat (D27–34). This decrease is undoubtedly the expression of the simplification of the bacterial flora of the intestinal tract, noted by Herter and Kendall in their experiments on kittens and monkeys.

The phenols and indican in these first experiments do not seem to be affected by changes in the type of protein in the food, except that after the indican had disappeared on a casein diet, traces again appeared in the urine when meat replaced the casein. In the second period of meat feeding, nitrogen excretion was distinctly smaller than in the first meat period, while the phenol to nitrogen ratio was larger than at any other time on this type of diet. Unfortunately it was not noticed whether this meat was especially tough or contained an unusual amount of indigestible connective tissue. This seems, however, the plausible explanation for the effect noted.

When beans were fed as the source of nitrogen, the indican again disappeared. The phenols underwent a small but clearly defined increase. This increase in phenol excretion was associated with a decided fall in the excreted nitrogen, although the amount of nitrogen in the food was the same as on the preceding meat diet. The decrease in nitrogen elimination means that an appreciable quantity of bean nitrogen was not being absorbed from the alimentary tract. With the increase in phenols and the decrease in total nitrogen there was a decided increase in the ratio of phenol to total nitrogen, so that it averaged nearly three instead of less than two, as on all other low nitrogen diets.

There was a distinct and immediate rise in phenol excretion when the nitrogen intake was increased two to three times. The ratio of total phenol to total nitrogen, however, remained at the same level as on the low nitrogen diets.

Gelatin.—From July 23 to August 5 the dog was kept on a diet in which gelatin was the source of nitrogen. It was impossible to induce the animal to take this diet with any degree of consistency and the attempt was finally abandoned. The daily

TABLE III.

Indican and Phenol Excretion in the Dog: Low Protein Diets.

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P : N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
D1	May 14	136 gm. meat.	432	5.1	0.24	91	60	31	66	34	1.8	1.8
D2	" 15		286	5.3	0.24	84	34	50	65	35	1.6	1.6
D3	" 16		306	5.4	0.18	104	64	40	63	37	1.9	2.3
D4	" 17		402	5.6	0.26	111	66	45	60	40	2.0	2.9
D5	" 18		316	5.3	0.24	82	53	29	65	35	1.5	1.4
D6	" 19		330	5.7	0.23	87	56	31	64	36	1.5	2.6
D7	" 20		325		0.23	89	50	39	55	45		1.5
D8	" 21		286	5.6	0.23	93	55	38	59	41	1.6	1.3
D9	" 22	18.7 gm. gelatin.	286	5.9	0.22	95	53	42	56	44	1.6	0.8
D10	" 23		250	5.6	0.29	94	64	30	68	32	1.7	1.5
D11	" 24		292	5.2	0.22	120	65	55	54	46	2.3	1.3
D12	" 25		234	5.2	0.21	86	48	37	57	43	1.7	0.1
D13	" 26		325	5.1	0.18	83	48	35	58	42	1.6	0.7
D14	" 27		272	5.3	0.23	75	44	31	59	41	1.4	2.2
D15	" 28		360	5.3	0.22	81	42	39	53	47	0.5	3.1
D16	" 29		248	5.0	0.23	79	48	31	61	39	1.6	0.5
D17	" 30		270	4.6	0.23	67	36	31	55	45	1.4	1.6
D18	" 31		252	4.6	0.22	71	38	33	54	46	1.5	3.3

D19	June 3	27 gm. casein.	248	4.6	0.21	84	54	30	64	36	1.8	0.8
D20	" 4		246	5.0	0.21	89	55	34	62	38	1.8	2.3
D21	" 5		149	3.8	0.23	86	50	36	59	41	2.2	None.
D22	" 6		200	5.0	0.20	85	56	29	66	34	1.7	"
D23	" 7		309	5.1	0.20	75	48	27	64	36	1.5	"
D24	" 8		238	4.7	0.22	75	46	29	61	39	1.6	"
D25	" 9		326	4.0	0.19	76	45	31	59	41	1.9	"
D26	" 10		380	4.3	0.10	74	50	24	67	33	1.7	"
D27	July 1	136 gm. meat.	312	3.3	0.20	65	53	12	82	18	2.0	1.0
D28	" 2		345	3.6	0.24	59	41	18	69	31	1.6	Trace.
D29	" 3		(?)	3.2	0.24	78	54	23	71	29	2.4	"
D30	" 4		228	3.4	0.24	84	61	23	71	29	2.5	"
D31	" 5		218	3.5	0.20	79	56	23	72	28	2.3	"
D32	" 6		412	3.6	0.22	84	62	22	75	25	2.3	"
D33	" 7		328	3.8	0.23	82	60	22	73	27	2.2	"
D34	" 8		282	4.0	0.25	71	55	16	77	23	1.8	"
D35	" 9	94 gm. beans.	166	3.1	0.23	88	59	29	67	33	2.8	None.
D36	" 10		176	3.1	0.23	88	52	37	58	42	2.8	"
D37	" 11		316	2.9	0.22	83	60	23	72	28	2.9	"
D38	" 12		270	2.8	0.23	85	52	23	71	39	3.0	"
D39	" 13		200	2.6	0.22	73	48	24	77	33	2.8	"
D40	" 14		270	2.8	0.24	82	42	40	51	49	3.0	"
D41	" 15		254	3.9	0.24	94	64	30	78	32	2.4	"

nitrogen elimination during this time ranged from 1.8 to 10.2 gm. The animal alternately fasted, or ate the gelatin. The two effects were hopelessly confused and these experiments are not reported in the tables.

Casein and Meat.—The experiments on meat feeding and on casein feeding confirm the results obtained with man.

The period of casein feeding was of longer duration than in our human experiments so that its effect has become more clearly established. The conclusion was the same, however. Indican and phenol were both excreted in appreciable amounts on this diet. The phenol as well as the indican output per day was distinctly higher on the meat diet. The effect of the meat diet on the physical condition of the animal was very marked. While on all other diets the animal was very lively and seemed contented, during the periods of meat feeding she became morose, stupid, and slept a large part of the time. When awake she kept chewing at the bars of the cage. After a few days the feces became black in color and tarry in consistency. By the 3rd or 4th day the animal either refused the food or, after eating, vomited. The diarrhea was undoubtedly a mechanism adapted to ridding the system of toxic materials.

Vegetable Proteins.—When glidine was fed in large amounts (Table IV, D67–71), the phenol and indican excretion attained about the same level as in the casein experiments. Indican experienced a sudden drop from 20 mg., on the last day of the preceding meat diet, to 5 mg. on the first glidine day. After this the amount of indican slowly fell to 1 mg. on the 5th and last day of the experiment. Unfortunately the animal refused to eat this diet any longer and subsequent attempts to carry out an experiment on this diet were not successful. We cannot, therefore, be certain whether indican excretion might not have ceased entirely. However, on all other diets, the level of indican excretion established on the 4th day was maintained throughout the period and there is no reason to believe the wheat proteins would have been an exception to this.

In contrast to the other vegetable protein, the bean protein which had been fed during the low nitrogen experiments, the wheat protein was fairly well absorbed as indicated by the urinary nitrogen. This is in agreement with the results of Mendel and

Fine (23). The ratio of total phenol to total nitrogen was at the usual level. In this case, then, there was no unusual amount of protein reaching the lower gut and causing enhanced activity of the putrefactive organisms, which would be followed by increased phenol excretion. This difference in the two vegetable proteins was merely the result of the fact that the bean protein was to a certain extent encased in an envelope of difficultly digestible material which prevented complete absorption. The wheat protein had been freed from this indigestible carbohydrate.

Carbohydrates.—Attempts were made to determine the effect of the addition of large amounts of carbohydrate to the high meat diets. In one experiment cracker meal was used, in the other, lactose. When the cracker meal and meat mixture was fed after a gliadine period, there was a definite increase in phenol excretion and also in indican excretion. The amount of these substances in the urine was not so large as was usually found in the urine on a meat diet, although the diet was fed for 5 days.

The lactose experiment was of shorter duration than would permit the drawing of clear-cut conclusions. As the result of the preceding 6 days fasting, phenol and indican had reached very low levels. The metabolism of the putrefactive organisms had decreased by reason of the small amount of metabolite. As soon as the meat and lactose diet was fed (Table IV, D86–88), the phenol and indican in the urine both increased. In order for the effect of the lactose in possible transformation of the flora to become evident a longer period of lactose feeding would obviously have been necessary.

When the meat and lactose diet was replaced by a diet containing meat alone (D89–91), there was an immediate sharp increase in both indican and phenol excretion. This would indicate that there may have been an inhibitory action from the lactose, due to the favoring of acidophilic organisms. However, when we compare the excretion of phenol and indican on the lactose period with the excretion of these substances during the preceding meat period (Table IV, D80–82), we see that there is really no distinct difference. Our results on carbohydrate feeding are suggestive rather than conclusive.

TABLE IV.

Indican and Phenol Excretion in the Dog: High Protein Diet; The Effect of Fasting.

Sample No.	Date.		Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P : N ratio.	Indican as indigo.
							Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919			*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
D42	July	16	82 gm. casein.	365	3.4	0.24	97	69	71	71	29	2.9	None.
D43	"	17		410	8.9	0.26	160	113	47	71	29	1.8	"
D44	"	18		410	10.0	0.26	140	93	47	66	34	1.4	1.9
D45	"	19		355	8.6	0.28	116	85	31	74	26	1.3	1.5
D46	"	20		410	9.9	0.24	152	104	48	69	31	1.5	2.1
D47	"	21		262	8.0	0.24	86	60	26	70	30	1.1	2.6
D48	"	22		275	8.5	0.25	91	62	29	68	32	1.1	3.3
D49	"	23	79 gm. gelatin.	650	10.3	0.21	86	37	49	57	43	0.8	4.2
D63	Aug.	6	417 " meat.	425	10.1	0.56	174	109	65	62	38	1.7	12.2
D64	"	7		315	9.4	1.25	302	178	124	59	41	3.2	53.2
D65	"	8		414	14.4	1.14	295	165	130	56	44	2.0	65.4
D66	"	9	Feces diarrheal.	282	9.8	0.91	164	101	63	62	38	1.7	19.8
D67	"	10	87.5 gm. glidine.	236	9.6	0.40	129	81	48	63	37	1.3	5.1
D68	"	11		220	12.6	0.41	134	89	45	67	33	1.1	4.2
D69	"	12		325	13.8	0.43	117	109	8	93	7	1.8	6.0
D70	"	13		335	12.1	0.38	141	95	46	67	33	1.2	2.3
D71	"	14	All food not taken.	214	7.2	0.43	133	95	38	71	29	1.8	1.0
D72	"	15	417 gm. meat, 165 gm. cracker meal.	356	12.4	0.81	172	127	45	74	26	1.4	5.8
D73	"	16		380	12.5	1.00	185	136	49	74	26	1.5	7.4

D74	Aug. 17		487	11.4	0.96	159	119	40	75	25	1.4	5.8
D75	" 18		250	22.0	0.78	163	115	47	71	29	0.7	3.0
D76	" 19	Vomits.	280	17.5	1.00	175	136	39	78	22	1.0	5.7
D77	" 20	Fasting.	164	4.9	0.44	107	76	31	71	29	2.2	6.5
D78	" 21		86	4.2	0.27	72	51	21	71	29	1.7	2.5
D79	" 22		77	2.7	0.28	73	53	21	73	27	2.7	2.7
D80	" 23	417 gm. meat.	202	7.9	0.86	126	100	26	79	21	1.6	5.5
D81	" 24		355	10.4	1.14	133	98	35	74	26	1.3	10.7
D82	" 25		540	9.0	0.98	132	94	38	71	29	1.5	12.2
D83	Sept. 2	Fasted since Aug. 29.	35	1.7	0.24	37	26	11	70	30	2.1	0.8
D84	" 4		45	2.2	0.24	39	30	9	77	23	1.8	None.
D86	" 6	417 gm. meat, 96 gm. lactose.	510	11.8	0.98	204	155	49	76	24	1.7	2.4
D87	" 7		375	10.0	0.79	181	128	53	71	29	1.8	5.7
D88	" 8	One-half food eaten.	320	8.6	0.72	165	112	53	68	32	1.9	19.9
D89	" 9	417 gm. meat.	310	3.8	0.95	248	147	101	59	41	6.5	35.9
D90	" 10		570	12.1	0.87	226	140	86	62	38	1.9	40.8
D91	" 11		282	14.3	0.71	266	161	105	61	39	1.8	31.7
D92	" 12	Fasting.	89	3.7	1.55	86	48	37	57	43	2.3	4.0
D93	" 13		72	3.2	0.28	61	50	11	82	18	1.9	2.7
D94	" 14		404	3.5	0.24	129	98	31	76	24	3.7	0.7

Fasting.—The results of inanition as seen in our experiments are in agreement with those of Dubin. There is a sharp decrease in the excretion of phenol, indican, and total nitrogen. The ratio of total phenol to total nitrogen does not change.

DISCUSSION.

When the results of Herter and Wakeman, of Rettger and his pupils, and of Torrey, who determined the effect of diet on the bacterial flora of the feces, are compared with our results on the effect of diet on the excretion of indican and the phenols, the relation between the two is established. Those diets which gave rise to the excretion of phenols and indican in large amounts were those diets which promoted the growth of the putrefactive microorganisms in the intestine. Thus a diet containing large amounts of meat wás found, by Herter and Wakeman, and by Torrey, to promote the growth of the putrefactive bacteria. Such a diet greatly stimulates the excretion of indican. The excretion of the phenols is also increased on this diet. The effect is not seen so clearly in man as in the dog, because in man there are wider variations in urine flow and the completeness of the emptying of the bowel.

Rettger and Torrey have shown that lactose is very efficacious in bringing about a predominance of the acidophiles. Torrey found that when starchy foods were fed there was a tendency toward elimination of the putrefactive types of bacteria. White bread and potatoes had this influence. On this type of diet, we have found that the excretion of phenols, especially, is lower than on any other type of diet.

Casein was found by Torrey to show a much smaller tendency to give rise to intestinal putrefaction than meat. We have found that this protein causes far less phenol and indican to appear in the urine than the meat diet.

Herter and Kendall found that, in the earlier stages of their experiments on kittens and monkeys, sudden shifts of diet were not accompanied by like changes in the character of the fecal flora. As their experiments were continued, however, sharper variations began to appear. They attributed this effect to the simplification of the flora caused by the elimination of all but the predominant putrefactive or acidophilic strains. Thus we have here, in the parallelism of our results with those of Herter and Kendall, another indication that variation in urinary indican and the phenols is associated with changes in the character of the intestinal flora.

Torrey found that the "Vegetable proteins stand in strong contrast to animal proteins, especially meat, in that they do not offer the slightest encouragement to the growth of intestinal putrefactive types of bacteria." Hull and Rettger, on the other hand, found that a "Diet of bread and vegetables does not encourage this flora (the acidophiles), but has the same influence as ordinary protein diet." In this instance our results follow those of Hull and Rettger rather than those of Torrey. For we have found that a mixture of wheat proteins was on the same level as the animal protein, casein, as far as the excretion of indican and phenol was concerned, and that the elimination of these substances was much higher on such a diet than when a diet containing a large amount of carbohydrate was fed.

It is very evident from our results that the determination of both indican and the phenols in the urine gives a much better indication of the extent of intestinal putrefaction than the determination of either of these substances alone. The extreme differences in different individuals in their tendency to excrete indican, especially, makes comparisons between individuals, in this respect, hazardous.

SUMMARY.

In a study of the urinary excretion of indican and the phenols, carried out on three normal individuals and on a dog, the following results were obtained:

1. The excretion of both indican and the phenols varies directly with the protein intake. In their smaller fluctuations, the excretion of phenol and indican does not necessarily vary in the same direction. In most cases, a *large* variation in indican excretion is accompanied by a variation in phenol excretion in the same direction.

2. In different individuals the phenol and indican excretion may bear quite a different relation to one another. A high indican excretion by an individual, as compared to other indi-

viduals, does not necessarily mean a correspondingly high excretion of phenols by that individual.

3. The effect of even mild constipation overshadows the effect of diet on the excretion of these substances. Constipation causes a large increase in the excretion of indican and the phenols. Diuresis, also, is shown to promote the excretion of these substances.

4. With increased phenol excretion there is stimulation of conjugation to the extent that in most cases the same *percentage* of the total phenol is excreted in conjugation.

5. Comparison of our results with those of Herter and Kendall, of Rettger and his pupils, and of Torrey indicates that those diets which promote the growth of the putrefactive bacteria also promote indican and phenol excretion.

(*a*) Meat ingested in large quantities causes a marked increase in the excretion of the phenols and indican.

(*b*) Casein causes a moderate excretion of indican and the phenols.

(*c*) Gelatin, containing no tryptophane building-stone, causes cessation of indican excretion with no change in the excretion of the phenols.

(*d*) Glidine, a mixture of wheat proteins, did not differ from casein in its effect.

(*e*) Kidney bean protein, fed with the usual admixture of indigestible carbohydrate substances, was less completely absorbed than the other proteins. Phenol excretion was increased by a small, but definite, amount.

(*f*) On a diet containing large amounts of lactose the excretion of indican and the phenols was lower than when the diet contained a large amount of protein.

6. In intelligent dieting with the regulation of intestinal putrefaction as the aim, it is necessary to study either the fecal flora or the excretion of phenols and indican.

BIBLIOGRAPHY.

1. Baumann, E., *Arch. ges. Physiol.*, 1876, xiii, 285.
2. Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.
3. Munk, I., *Arch. ges. Physiol.*, 1876, xii, 142.
4. Kossler, A. V., and Penny, E., *Z. physiol. Chem.*, 1893, xvii, 117.
5. Hensel, M., *Z. physiol. Chem.*, 1912, lxxviii, 373.
6. Herter, C. A., and Kendall, A. I., *J. Biol. Chem.*, 1909–10, vii, 203.
7. Rettger, L. F., and Horton, G. D., *Centr. Bakt.*, *1te Abt.*, *Orig.*, 1914, lxxiii, 362.
8. Hull, T. G., and Rettger, L. F., *Centr. Bakt.*, *1te Abt.*, *Orig.*, 1914, lxxv, 219.
9. Rettger, L. F., *J. Exp. Med.*, 1915, xxi, 365.
10. Torrey, J. C., *J. Med. Research*, 1918–19, xxxix, 415.
11. Torrey, J. C., *J. Infect. Dis.*, 1915, xvi, 72.
12. Hirschler, A., *Z. physiol. Chem.*, 1886, x, 306.
13. Underhill, F. P., *Am. J. Physiol.*, 1904–05, xii, 176.
14. Homer, A., *J. Biol. Chem.*, 1915, xxii, 351.
15. Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.
16. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.
17. Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 507.
18. Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69.
19. Dubin, H., *J. Biol. Chem.*, 1916–17, xxviii, 429.
20. Moore, C. U., *Am. J. Dis. Child.*, 1917, xiii, 16.
21. Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 17.
22. Ellinger, A., *Z. physiol. Chem.*, 1903, xxxviii, 178.
23. Mendel, L. B., and Fine, M. S., *J. Biol. Chem.*, 1911–12, x, 303.
24. Woods, C. D., and Merrill, L. H., *U. S. Dept. Agric.*, *Office of Experiment Stations, Bull. 85*, 1900.

BIOCHEMICAL STUDIES ON MARINE ORGANISMS.

I. THE OCCURRENCE OF COPPER.

By WILLIAM C. ROSE and MEYER BODANSKY.

(From the Laboratory of Biological Chemistry of the School of Medicine, University of Texas, Galveston).

(Received for publication, August 2, 1920.)

INTRODUCTION.

The occurrence of copper in tissues of certain marine animals has been known for a long time. More than 70 years ago Harless (1847) detected copper in the blood of *Eledone* and *Helix pomatia*, and showed that it exists not as a free salt, but in combination with protein. This peculiar copper-containing protein, which was subquently named hemocyanin by Frédéricq (1878), is apparently an oxygen carrier analogous to the hemoglobin of higher animals. It differs in locality from the latter in occurring in the plasma rather than in the form elements of the blood. Unlike hemoglobin it cannot be disintegrated into a protein and a protein-free component. When shaken with air, hemocyanin combines loosely with oxygen, and is converted into blue oxyhemocyanin, and by the escape of the oxygen becomes colorless again. According to Henze (1901), hemocyanin of the *Octopus* may be crystallized, and the crystalline preparation has the following composition: C = 53.66; H= 7.33; N = 16.09; S = 0.86; O = 21.67; and Cu = 0.38 per cent.

Abundant evidence has been adduced to prove that hemocyanins of different sources are not identical in composition or physical properties. In a series of investigations Dhéré (1900, 1903, 1913, 1914, 1919) examined the blood of various mollusks and arthropods, and noted peculiarities in crystallization, percentage of copper, and color reactions of the respective hemocyanins. He states that the efficiency of a hemocyanin as an oxygen carrier is directly proportional to its copper content.

This latter observation is of considerable physiological interest. In many cases at least, the more active species requiring a rapid respiratory exchange are provided with blood containing larger percentages of copper than other species of a low metabolic type. This fact was emphasized by Mendel and Bradley (1906–07). Thus the blood of the highly active *Octopus* contains 18 to 23.5 mg. of copper per 100 cc., while the very inactive *Helix pomatia* is provided with only 6.5 to 12.5 mg. per 100 cc. More recently Alsberg and Clark (1910–11) have made a careful investigation of the hemocyanin of *Limulus polyphemus*, and find that it differs materially from the preparation studied by Henze. *Limulus* hemocyanin contains 0.28 per cent of copper, is much richer in sulfur and oxygen, and contans less carbon than the respiratory protein of *Octopus*. The copper in Alsberg and Clark's preparation was in an exceedingly loose combination, which could be completely removed by dialysis if the water used were kept very faintly acid.

One of the most interesting differences in hemocyanins is that noted by Mendel and Bradley (1905, 1906–07) in the case of the marine gastropod, *Sycotypus canaliculatus*. The respiratory protein of this mollusk was found to contain zinc in addition to copper. The name "hemosycotypin" was suggested for this peculiar oxygen carrier. Numerous other investigators, notably Lankester (1872–73), Krukenberg (1880), Halliburton (1885), Griffiths (1890–91, 1892), and Philippi (1919), have studied hemocyanins of various sources. The earlier investigations are discussed in detail in the excellent review of von Fürth (1903). The fact which we wish to emphasize here is the wide occurrence of copper as a constituent of invertebrate blood. Nor is this metal confined to the circulating fluid, but may occur in other portions of the body as well. Through the investigations of Herdman and Boyce (1898–99), Nelson (1915), and others, copper is known to occur in relatively large and variable amounts in oysters. According to Willard (1908), and Hiltner and Wichmann (1919), this metal may be present in sufficient amount to impart a greenish tint to the oysters, or even to produce toxic symptoms when they are ingested by man.

In Table I are listed the animals in which copper is known to occur, either as hemocyanins in the blood, or stored in unknown combinations elsewhere in the organism.

TABLE I.

Animals in Which Copper is Known to Occur.

Animal.	Part analyzed.	Reference.
Echinodermata.		
Asterias.........	Entire animal.	Dubois (1900).
Echinus.........	“ “	“ (1900).
Stichopus.......	“ “	“ (1900).
Holothuria......	“ “	Phillips (1917).
Annulata.		
Hirudo..........	“ “	Dubois (1900).
Crustacea.		
Palaemon.......	“ “	“ (1900).
Clibanarius	“ “	“ (1900).
Astacus.........	Blood.	Griffiths (1890–91).
Squilla..........	“	Krukenberg (1880).
Palinurus.......	“ and liver.	Griffiths (1890–91); Phillips (1917).
Nephrops.......	“	Halliburton (1885).
Homarus........	“	“ (1885); Griffiths (1890–91).
Carcinus........	“	Griffiths (1890–91).
Maja...........	“	Kobert (1903).
Eriphia.........	“	Krukenberg (1880).
Cancer..........	“ and liver.	Harless (1847); Griffiths (1890–91).
Arachnida.		
Scorpio.........	“	Lankester (1872–73).
Limulus.........	“	“ (1872–73); Alsberg and Clark (1910–11).
“	Liver, ovaries, etc.	Phillips (1917).
Pelecypoda.		
Ostrea...........	Entire animal.	Herdman and Boyce (1898–99). Willard (1908). Hiltner and Wichmann (1919).
Anodonta.......	Blood.	Griffiths (1890–91).
Mytilus.........	“	“ (1890–91).
Pinna...........	“	“ (1890–91).
Mya............	“	von Fürth (1903).
Pecten..........	Entire animal.	Dubois (1900).
Unio............	“ “	“ (1900).
Gastropoda.		
Helix...........	Blood.	Harless (1847).
Murex..........	“	Kobert (1903).
Turbo...........	“	“ (1903).

TABLE I—*Concluded.*

Animal.	Part analysed.	Reference.
Aplysia.......	Liver.	Phillips (1917).
Fissurella.....	Blood.	Kobert (1903).
Haliotis.......	"	" (1903).
Sycotypus.....	" and liver.	Mendel and Bradley (1905).
Fulgur..........	Liver.	Bradley (1904); Phillips (1917).
Cassidaria......	Blood.	Kobert (1903).
Fasciolaria......	Liver.	Phillips (1917).
Cassis..........	"	" (1917).
Strombus........	Soft parts.	" (1917).
Triton.... . ..	Blood.	von Fürth (1903).
Arion...........	"	" " (1903).
Cyclostoma......	"	" " (1903).
Scaphander......	"	" " (1903).
Paludina........	"	" " (1903).
Cephalopoda.		
Loligo...........	"	Kobert (1903).
Sepia...........	"	Griffiths (1890–91).
Octopus.........	"	Frédéricq (1878); Henze (1901).
Eledone.........	" and liver.	Harless (1847); Dhéré (1913).
Tunicata.		
Ciona...........		Phillips (1917).
Elasmobranchii.		
Acanthias.......	Liver.	Harless (1847).
Teleostomi.		
Zeus............	"	" (1847).
Conger..........	"	" (1847).

As will be observed, the data thus far accumulated are chiefly confined to arthropods and mollusks, in which copper is invariably present. As discovered by Mendel and Bradley (1905), zinc is likewise present in relatively large amounts in at least one form of mollusks (*Sycotypus*), though the examination of various other marine animals—*Mytilus edulis*, *Modiola plicatula*, *Argina pexata*, *Eupagurus pollicaris*, *Ostrea virginiana*, and *Cancer irroratus*—failed to reveal appreciable amounts of this metal. According to these investigators, traces of zinc seemed to be present usually in *Urosalpinx cinerea*. In the liver of *Sycotypus* copper comprises about 8 per cent of the total ash, or 1.2 per cent of the dry tissue; while zinc comprises about 15 per cent of the ash, or 1.7 per cent of the dry tissue.

Because of the incompleteness of the data on the occurrence of copper and zinc in many forms of marine life, and the ease with which these animals may be procured in this locality, we have undertaken a quantitative study of their copper and zinc content. We have confined our work chiefly to fishes, with a few comparative data upon cœlenterates, mollusks, and arthropods. In the present paper we present the copper findings only, and will report the data upon zinc in a subsequent communication.

Methods.

In the present work copper was estimated in specimens prepared by thoroughly grinding the entire animals soon after they were removed from the water. The fishes were hashed without preliminary evisceration or scaling, and the crabs and shrimps without removal of the protective coverings. The oysters and clams were shucked. We expect to attempt detailed examinations of the tissues and organs in certain species later, but preliminary to subsequent investigations we desired comparative information concerning the copper content of the entire individuals.

In the estimation of copper we have tried several procedures, and finally adopted a colorimetric method based upon the familiar "blue test" with an excess of ammonia. A similar procedure was recommended by Heath (1897) in the analysis of tailings and lean blast furnace slags, but we have found it necessary to modify the conditions suggested by him to meet our special needs. Despite the fact that the method is an exceedingly long and tedious one, we have used it in preference to a shorter gravimetric or electrolytic process because of the difficulty of accurately weighing such small quantities of copper as those with which we have had to deal. It has been our experience that great care must be taken in following every detail of the procedure as adopted by us, in order to avoid the introduction of errors. We therefore describe the method in full without claiming any originality for it.

Preparation of Standard Copper Solution.—About 2 gm. of electrolytic copper are washed in dilute nitric acid to remove the trace of oxide, and then successively in water, alcohol, and ether. The dry metal is accurately weighed, and dissolved in 10 to 15 cc. of concentrated nitric acid. 5 cc. of concentrated sulfuric acid are added, and the solution is heated on the water bath until the nitric acid is entirely removed. The solution is then diluted with about 200 cc. of distilled water, ammonia cautiously added until the reaction is just faintly acid, and the clear blue preparation diluted until each cubic centimeter contains exactly 1 mg. of copper.

Oxidation of the Tissue.—The weighed material (usually 100 gm.) is introduced into a porcelain dish or casserole, treated with 20 cc. of concentrated sulfuric acid, and slowly evaporated on a sand bath to a crisp charred mass, care being taken to prevent loss through spattering. The dry residue is allowed to cool, and treated with concentrated nitric acid, using the latter to wash down the sides of the vessel. 5 to 10 cc. of nitric acid are usually sufficient for the purpose. The vessel is again subjected to careful heating until the nitric acid is driven off. If necessary, this process is repeated two or three times, or until the quantity of charred material is considerably reduced, and may be transferred to a large (80 to 90 cc.) crucible.

This transfer is made as follows: the major portion of the material is carefully scraped into the crucible with a glass rod, the dish washed with the minimum quantity of dilute nitric acid, and the last traces of charred residue are carefully wiped from the sides of the dish with a small piece of ash-free filter paper. The washings and paper are added to the contents of the crucible, and the whole is evaporated to dryness on a water bath. The crucible is baked for a few minutes on a sand bath to remove the last traces of nitric acid, and ignited over a Bunsen burner at a dull red heat until a white or gray ash (blue if much copper is present) is obtained. If the charred material is large in amount, it is convenient to ash directly that which is scraped from the dish, and to add the washings and filter paper afterwards with subsequent evaporation and ignition. Occasionally, concentrated nitric acid must be added two or three times toward the last of the operation in order to secure complete oxidation of the organie matter. In this manner no difficulty has been experienced in obtaining an ash free from organic material.

Precipitation and Estimation of Copper.—The ash prepared as described above is extracted repeatedly with small quantities of hot dilute hydrochloric acid, the extracts are poured through a good ashless filter paper, and the residue and paper are thoroughly washed with boiling water. If the combined filtrate and washings exceed 50 cc., the solution is evaporated to approximately this volume. Otherwise it is neutralized directly with ammonium hydroxide, and again acidified by the addition of 1 or 2 cc. of concentrated hydrochloric acid.[1] A strong current of hydrogen sulfide is passed into the hot solution for 10 to 15 minutes, and the flask heated on the water bath until the sulfide precipitate settles. The latter is rapidly filtered off and washed with dilute hydrochloric acid (approximately 0.2 N in strength) which has previously been saturated with hydrogen sulfide.

---

[1] In the analysis of certain marine animals, notably those having a chitinous protective covering, the ash contains a large quantity of calcium sulfate which is partly dissolved by the repeated acid extractions. Under these conditions a precipitation of calcium sulfate not infrequently occurs when the solution is concentrated, or neutralized and reacidified with hydrochloric acid. This necessitates an additional filtration before the sulfide precipitation.

The filter paper containing the sulfide precipitate is returned to the original flask in which the precipitation occurred, and repeatedly boiled with small portions of 2 N nitric acid to dissolve the copper. Each extract is decanted through a filter paper, the residue finally transferred to the paper, and washed with boiling water until the washings are neutral in reaction. The combined filtrate and washings are evaporated to dryness in a small casserole, and the residue is baked for a few minutes at a dull red heat to oxidize the trace of organic matter (or sulfur) which at this point is almost invariably present. The casserole is allowed to cool, and the cupric oxide dissolved in a few drops of concentrated nitric acid. A perfectly clear blue solution should result. If unoxidized matter is still present, as indicated by a slight yellow or brown color in the solution, the evaporation and baking must be repeated. Occasionally this process must be repeated three times.

When the copper nitrate is free from interfering matter, it is treated with 1 to 2 cc. of concentrated sulfuric acid, and carefully heated until white fumes are given off. The copper sulfate so obtained lends itself readily to the colorimetric estimation. For this purpose the contents of the casserole are diluted with a few cubic centimeters of distilled water, and washed into a 50 cc. Nessler jar. 20 cc. of strong ammonium hydroxide are cautiously added,[2] the solution is diluted to the 50 cc. mark, and mixed. The copper content is determined by running the standard copper sulfate solution into another Nessler jar containing 2 cc. of concentrated sulfuric acid, 20 cc. of distilled water, and 20 cc. of strong ammonium hydroxide, until the two solutions manifest exactly the same depth of color when examined longitudinally against a white background. The burette reading indicates the milligrams of copper in the sample taken for analysis.

If the copper content of the sample is greater than 8 mg., it is advisable to dilute the contents of the Nessler jar to a suitable volume with ammonia (1 volume of strong ammonium hydroxide to 3 volumes of distilled water), and use an aliquot part for the titration. Two closely agreeing titrations should be made against each unknown.

Analyses of aqueous copper solutions carried through the entire process, and of ground meat to which known amounts of copper were added, indicate that the maximum error in the use of the method is approximately 5 per cent, as indicated in Table

---

[2] The large excess of ammonium hydroxide is necessary to produce a clear permanent blue color, rather than the purple or purplish blue which results with smaller amounts of the base.

II. This is about as small an error as could be expected in the estimation of such traces of copper, especially in view of the fact that the destruction of the tissue, which is necessary in any procedure, is the most probable source of loss.

TABLE II.

Recovery of Copper from Known Mixtures.

Experiment No.	Material.	Cu present.	Cu added.	Total Cu.	Cu found.
		mg.	*mg.*	*mg.*	*mg.*
1	100 cc. of distilled water.	0	5.0	5.0	5.0
2	100 " " " "	0	10.0	10.0	10.0
3	50 gm. of meat.*	0	5.0	5.0	4.85
4	50 " " "	0	10.0	10.0	10.2
5	50 " " "	0	20.0	20.0	20.8
6	50 " " "	0	2.0	2.0	1.95
7	50 " " "	0	16.0	16.0	15.6
8	100 " of oysters.†	4.52	3.0	7.52	7.1
9	100 " " " ‡	6.00	5.0	11.0	10.7

* Preliminary analysis showed that 50 gm. of the meat did not contain a detectable amount of copper.

† No. 2 of Table III.

‡ No. 4 of Table III.

EXPERIMENTAL.

Using the above method, we have determined the copper in about thirty-five specimens of marine animals, the results of which are tabulated in Table III. The figures recorded are in most cases the averages of closely agreeing duplicates. With the exception of two analyses, one upon a large variety of clam, and the other upon a pigfish, copper has been found present in every case. Where we were unable to detect copper, the amount of tissue available for analysis was rather small, and we have been unable to procure other specmens of these species for duplicate determinations.

Of the fishes, the common sea-catfish contained the least copper, only minute traces (less than 1 mg. per kilo) being found in any of the three specimens analyzed. One sample of croaker (No. 14) showed less than 1 mg. of copper per kilo of tissue, while a second specimen (No. 26) showed 2.5 mg. per kilo. In

TABLE III.

Quantity of Copper in Marine Animals.

Analysis No.	Animal.	Amount taken for analysis.	Cu found.	Cu per kilo.	Remarks.
		gm.	*mg.*	*mg.*	
	Cœlenterata.				
9	Jellyfish.....	200	0.30	1.5	Entire animal ground.
42	Portuguese man-of-war.......	272	0.60	2.2	One entire animal.
44		81	0.30	3.7	Two small individuals.
	Pelecypoda.				
2	Oysters......	100	4.52	45.2	Shucked.
4	"	100	6.00	60.0	
31	"	100	4.30	43.0	
32	"	100	2.40	24.0	
37	"	100	4.70	47.0	
39	Clams..	77	0.0	0.0	
	Crustacea.				
1	Shrimps......	100	1.70	17.0	Includes the protective coverings.
49	"	100	1.40	14.0	
3	"	100	0.80	8.0	
5	Crabs........	100	0.65	6.5	
10	"	100	0.50	5.0	
	Elasmobranchii.				
29	Torpedo ray.	100	0.35	3.5	Three ground and sampled.
16	Sting ray....	100	0.52	5.2	Entire animal ground.
	Telestomi.				
6	Mullet.......	100	0.28	2.8	One or more individuals thoroughly ground and mixed before sampling.
15	"	100	0.20	2.0	
19	Angelfish.....	59	0.25	4.2	
11	Sea-catfish...	100	Trace.	Trace.	
20	" ...	100	"	"	
24	" ...	100	"	"	
14	Croaker......	100	"	"	
26	"	100	0.25	2.5	
23	Pigfish.......	43	0.0	0.0	
25	Whiting......	100	0.10	1.0	
28	Yellow-tail...	94	0.25	2.7	
12	Flounder.....	100	0.22	2.2	
27	"	100	0.25	2.5	
18	Spotted trout.	100	0.18	1.8	
17	Redfish......	100	0.20	2.0	
21	Drum........	86	0.25	2.9	
22	"	100	0.25	2.5	
30	Tarpon......	100	0.24	2.4	

general the fishes contain a low but rather constant amount of copper, averaging about 2.5 mg. per kilo of tissue. The two species of cœlenterates—the common jellyfish and the Portuguese man-of-war—contain about the same proportion of copper in the fresh tissue as do the fishes, though the tissues of the former have a much higher water content. On a dry weight basis the cœlenterates would manifest a much higher copper content than the fishes.

Oysters were by far the richest in copper of the animals examined, the content varying from 24 to 60 mg. per kilo. These figures are, however, considerably lower than those obtained by Nelson (1915), who found that blue oysters contained 40 mg. of copper each, and that uncolored ones contained 9 mg. each. In their extended study of the copper content of these mollusks, Hiltner and Wichmann (1919) report figures ranging from 6 to several hundred mg. per kilo, and state that oysters grown in water highly contaminated with metallic wastes from smelters and other factories contain the larger proportions of this metal. These authors are inclined to believe that the copper exists in the blood of the oyster, as in other mollusks, since the blood-containing fluid drained from shucked oysters invariably shows the presence of copper. Herdman and Boyce (1898–99) have demonstrated by microchemical tests that in certain oysters copper may occur in the leucocytes.

We have made one quantitative determination of the distribution of copper in fresh oysters. For this purpose 300 gm. were separated by careful dissection into four fractions composed of (*a*) mantles, (*b*) digestive glands or hepatopancreases, (*c*) adductor muscles, and (*d*) the other parts including the gills, which we have called the "residue." These fractions were weighed and analyzed separately, the results of which are shown in Table IV. The data indicate that the copper is rather uniformly distributed. No marked differences in the four fractions were found except in the muscle, which contained only about half as much copper as the other tissues. The residue was somewhat higher than the fractions composed of digestive organs and secreting mantles, possibly due to the vascularity of the gills which were contained in this fraction.

To determine whether copper exists in oysters in a diffusible form, the following experiment was made. A sample (87.5 gm.) of oysters, No. 37, containing 4.1 mg. of copper, was thoroughly ground, preserved with toluene, and dialyzed against distilled water. After 26 hours the dialysate was removed, and the experiment continued with fresh distilled water for 70 hours. Approximately 3 liters of water were used in all. Analysis of the combined dialysates showed that 0.5 mg., or 12.2 per cent of the total copper, had diffused. Hiltner and Wichmann (1919) believe that oysters store copper in excess of their physiological needs. A similar opinion is held by several investigators concerning the storage of copper in the livers of gastropods. Perhaps the diffusible metal in our specimen represented the stored surplus; or it may be that the copper exists ordinarily in a very loose combination with protein, such as was found to be the case in the hemocyanin of *Limulus* by Alsberg and Clark (1910–11).

TABLE IV.

Distribution of Copper in Oysters.

Tissue.	Amount taken for analysis.	Cu found.	Cu per kilo.
	gm.	*mg.*	*mg.*
Mantles	84.0	3.00	35.7
Digestive glands	56.7	1.95	34.4
Adductor muscles	38.5	0.70	18.2
Residue	102.0	4.10	40.2
Total	281.2*	9.75	34.7

* 300 gm. of fresh oysters were used for this experiment, but small fragments of tissue were unavoidably lost during the dissections, and considerable evaporation of water occurred. These factors account for the decrease of 18.8 gm. in weight.

As shown in Table III, the shrimps and crabs had copper contents intermediate between oysters and fishes, the shrimps averaging about twice as much copper as the crabs, though differing considerably in the three samples examined. In a distributional test upon shrimps, the protective coverings were removed from 200 gm. and the hulls (including the heads and tails), and the meats were analyzed separately. The hulls amounted to 89.0 gm. and the meats to 104.5 gm., 6.5 gm. of water having been lost through evaporation during the hulling. Contrary to our expectation, the hulls showed 7.3 mg. of copper per kilo, while the meats contained only 2.4 mg. per kilo, indicating that most of the copper in the shrimp is located in the protective covering.

DISCUSSION.

We believe that the data presented above clearly indicate that copper is a normal—possibly an essential—constituent, not only of mollusks and arthropods, where it is known to serve as an important ingredient of the respiratory protein, but of the tissues of fishes as well. Whether it exists in the latter as hemocyanins, or as other compounds of physiological importance, cannot at present be stated. It is interesting to observe that in the organisms examined by us the amounts of copper progressively diminish as we ascend from the comparatively simple to the more complex forms of life, and that when we come to the fishes, which are provided with hemoglobin, copper persists at a low but rather constant level.

In view of the almost universal occurrence of copper in marine animals it is not difficult to understand its source in fishes. Living largely at the expense of crustaceans, mollusks, and other low forms, fishes have an abundant supply of the metal in their diets. We believe that the universality of its occurrence and the constancy of its amount speak against its being an accidental constituent, or one due to the trace of copper in sea water. Hiltner and Wichmann (1919) report two analyses of sea water made by the Bureau of Chemistry laboratory at New York. In one, no copper could be detected in 3,500 cc.; in the other, a trace was detected in 4,000 cc. We have analyzed a specimen of water obtained about 30 feet from shore along the bathing beach of Galveston, where the maximum contamination would prevail, and found 0.14 mg. of copper per kilo of water. This figure is about one-twentieth of the average copper content of fishes. In a later communication we hope to present data on the distribution of this metal in some of the larger species.

SUMMARY.

1. Copper has been found to occur in two forms of cœlenterates; namely, the common jellyfish (*Aurelia*), and the Portuguese man-of-war (*Physalia*).

2. In oysters obtained locally, copper is rather uniformly distributed throughout the tissues as indicated by separate analyses of the mantle, digestive gland (hepatopancreas), large adductor muscle, and the remaining parts (residue) including the gills. The chief difference in the proportion of copper occurs in the muscle, where only half as much of the metal is found as elsewhere in the organism. Part of the copper in oysters (12.2 per cent in our experiment) exists in a diffusible form.

3. Analyses of twenty specimens of common fishes showed the presence of copper in every case except one, and the failure to detect it in this case was probably due to the small amount of material available for analysis. The average copper content was approximately 2.5 mg. per kilo.

4. It is believed that copper is a normal—possibly an essential—constituent of marine animals, and not an accidental one due to the trace of this metal in sea water.

BIBLIOGRAPHY.

Alsberg, C. L., and Clark, E. D., *J. Biol. Chem.*, 1910–11, viii, 1.

Bradley, H. C., *Science*, 1904, xix, 196.

Dhéré, C., *Compt. rend. Soc. biol.*, 1900, lii, 458; 1903, lv, 1161.

Dhéré, C., *Compt. rend. Acad.*, 1913, clvii, 309.

Dhéré, C., *J. physiol. path. gén.*, 1914, xvi, 985.

Dhéré, C., *J. physiol. path. gén.*, 1919, xviii, 222.

Dubois, R., *Compt. rend. Soc. biol.*, 1900, lii, 392.

Frédéricq, L., *Bull. Acad. roy. Belg.*, 1878, xlvi, Series 2, No. 11.

von Fürth, O., Vergleichende chemische Physiologie der niederen Tiere, Jena, 1903.

Griffiths, A. B., *Proc. Roy. Soc. Edinburgh*, 1890–91, xviii, 288.

Griffiths, A. B., *Compt. rend. Acad.*, 1892, cxiv, 496.

Halliburton, W. D., *J. Physiol.*, 1885, vi, 300.

Harless, E., *Arch. Anat., Physiol. u. wissensch Med.*, 1847, 148.

Heath, G. L., *J. Am. Chem. Soc.*, 1897, xix, 24.

Henze, H., *Z. physiol. Chem.*, 1901, xxxiii, 370.

Herdman, W. A., and Boyce, R., *Rep. Thompson-Yates Lab.*, 1898–99, ii, suppl. 1.

Hiltner, R. S., and Wichmann, H. J., *J. Biol. Chem.*, 1919, xxxviii, 205.
Kobert, R., *Arch. ges. Physiol.*, 1903, xcviii, 411.
Krukenberg, C. F. W., *Centr. med. Wissensch.*, 1880, xviii, 417.
Lankester, E. R., *Proc. Roy. Soc. London*, 1872–73, xxi, 71.
Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1905, xiv, 313.
Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1906–07, xvii, 167.
Nelson, J., *New Jersey Agric. Exp. Station, Ann. Rep.*, 1915, xxxvi, 246.
Philippi, E., *Z. physiol. Chem.*, 1919, civ, 88.
Phillips, A. H., *Carnegie Inst. of Washington, Publ. 251*, 1917, xi, 91.
Willard, J. T., *J. Am. Chem. Soc.*, 1908, xxx, 902.

A STUDY OF THE FACTORS WHICH INTERFERE WITH THE USE OF YEAST AS A TEST ORGANISM FOR THE ANTINEURITIC SUBSTANCE.

By GERALDO DE PAULA SOUZA AND E. V. McCOLLUM.

(*From the Laboratory of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.*)

(Received for publication, July 14, 1920.)

In a recent paper Williams[1] described a method of testing for the presence of the antineuritic substance which in this laboratory has been generally referred to as water-soluble B. For this purpose yeast was employed, and the basis of the test was the assumption, verified by experimental trials by Williams, that the yeast cell is capable of multiplication only when the antineuritic substance is present in its nutrient medium. By means of a series of dilutions of solutions which he used for the cultivation of yeast he was able to obtain results which gave promise of his method being useful in a quantitative sense as well as qualitative. Owing to the fact that the test as described by Williams could be carried out with very small amounts of material, and in a period of time much shorter than is required for the demonstration of the presence of the antineuritic substance in a nutrition experiment on a mammal, the method proposed for utilizing yeast as an experimental organism seemed to offer great possibilities of usefulness to those who are engaged in the study of methods for the isolation and identification of this most interesting dietary factor.

The work reported in this paper had its origin in an effort to develop proficiency in the conduct of experiments with yeast by the Williams method.

Considerable inconvenience was experienced in carrying out the test, owing to the number of small particles which became detached from the pen, and which interfered with the counting of the number of cells. A modification of Williams' method was therefore introduced, in which a platinum syringe needle, having an opening at right angles to its axis, was used for depositing the droplets on the cover-slip. The needle was attached to a piece of glass tubing by means of a short piece of rubber tubing, and it was found that with this instrument the suspension of yeast cells could be easily and quickly distributed, without leaving any detritus in the droplets. It was found that a fairly uniform distribution of cells could be obtained by this method. The slides were incubated in the manner described by Williams.

---

[1] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

Our results were characterized by lack of uniformity in the development of the yeast, and for this reason an attempt was made to substitute for the cover-slips used by Williams the ordinary blood counting chamber of the Levy type. In order to break up the clumps of yeast cells without injuring their vitality air was blown through the suspension instead of shaking the latter. The solution was then distributed in test-tubes, 5 or 10 cc. in each. The results by this technique were much more uniform. Table II shows on one hand how the cells frequently were distributed in the droplet method, and on the other how the same suspension behaved in the blood counting chamber method.

Williams called attention in his paper to the fact that many times it is impossible to count the number of cells after a period of growth on account of the fact that the cells grow in three dimensions. He found it necessary to count a part and estimate the probable number. By the counting chamber method it is not necessary to guess at the number of cells. If the suspension is too rich it may be diluted to such a point as facilitates the counting. Immediately before counting, a little formalin was added to the solution in order to kill the cells and avoid further growth. Using this technique we have attempted to test several substances known to be rich or poor in the dietary factor water-soluble B. We will first consider the results obtained by the extraction of wheat germ with hot water.

1 gm. of wheat germ was boiled with 50 cc. of water for 10 minutes (Extract 1), the residue was boiled again with 50 cc. of water for 10 minutes (Extract 2), and the second residue was boiled with 50 cc. of water for 10 minutes (Extract 3). The separation of the residue from the soluble part offered some difficulty due to the viscosity of the solution. In some cases we

have separated the solution from the undissolved part by means of a centrifuge and in others by filtration in an alundum crucible. Only by passing the solution through a Berkefeld filter could an approximately clear solution be obtained. Eight successive extractions were made.

These extracts were evaporated to the volume of 25 cc. so each 1 cc. corresponded to 0.04 gm. of wheat germ. 5 cc. of the control solution were put into test-tubes previously sterilized, and the first extract was added as shown in Table I.

TABLE I.

Tube No.	Control solution.	First extract.	Control solution.
	cc.	*cc.*	
1	5.0	0	Seeded with Fleischmann's yeast.
2	5.0	0.1	
3	5.0	0.2	
4	5.0	0.3	
5	5.0	1.0	

From these tubes we made a series of slides by the droplet method and ran a comparative series by the counting chamber method, the results of which are presented in Table II.

If we take into account only those droplets which contain one single cell, we observe that they give rise to very different numbers of cells by the end of an 18 hour incubation period. In those squares where the yeast cell was dead, or had reached the stage where budding had ceased, there was, of course, no multiplication.

Seeding 1.

1 cell gave rise to 3 cells.
1 " " " " 14 "
1 " " " " 27 "
1 " " " " 34 "
1 " " " " 62 "

Seeding 2 (Control Solution Plus 0.1 cc. of Wheat Germ Extract).

1 cell gave rise to 3 cells.
In the rest the single cells were dead or incapable of budding.

TABLE II.

*Yeast Cells Counted in Different Droplets.**

Tube 1.					Tube 2.					Tube 3.					Tube 4.					Tube 5.				
Before incubation.																								
1	2	**1**	0	0	0	**1**	2	2	**1**	**1**	2	**1**	**1**(?)	0	2	**1**	**1**	0	0	1	D†	**1**	0	0
D	3	0(?)	0	0	3	2	D(?)	3	2	2	**1**	3	2	0	3	**1**	2	2	D	0	2		3	0
1	2	2	D(?)	**1**	**1**	**1**	**1**	0(?)	2	**1**	2	2	0	0	2	**1**	**1**	2	**1**	0	**1**	2	**1**	3
D	**1**	**1**	**1**	**1**(?)	**1**	2	2	0	**1**	2	3	0	**1**	2	3	0	**1**	0(?)	**1**	0	0	**1**	2	**1**
0	**1**	**1**	**1**(?)	**1**	0	3	2	**1**(?)		**1**	**1**	2	**1**	2	4	0	**1**	0	0	**1**	2(?)	**1**	2	0
After incubation for 18 hrs.																								
1	500	**0**	0	0	0	**1**	0	130	0	**207**	252	**301**	**0**	0	2	**1**	**245**	0	0	**500**	500	**1**	0	
27	3	2	0	0	0	2	143	6	98	258	**1**	0	0	0	0	**1**	2	500	0	0	5			
1	2	0	0	0	**1**	**1**	0	254	143	**101**	2,000	0	0	0	2	**1**	**0**	1,000	**1,000**	0	**0**	342	**1,000**	500
19	**1**	**3**	**62**	**0**	**3**	2	31	0	**0**	2	290	0	**151**	1,500	440	0	**130**	500	**349**	0	0	**1,000**	0	**290**
0	**3**	**27**	**34**	**14**	0	0	41	**0**		**0**	**124**	394	**0**	300	1,000	0	**400**			**500**		**75**		0

* In those cases where we were successful in seeding with a single cell the entry is made in bold-faced type. In the table showing the growth of the cells, the bold-faced type represents the progeny of single cells.

† D = Dead.

Seeding 3.

1 cell gave rise to 101 to 301 cells.
2 cells " " " 252 " 2,000 "

Seeding 4.

1 cell gave rise to 130 to 1,000 cells.

Seeding 5.

1 cell gave rise to 75 to 1,000 cells.

On the other hand, when using the test-tubes which contain 5 to 10 cc. of control solution, to which different amounts of wheat germ extract were added as stated above, by using the counting chamber we have a greater chance of securing the same number of cells distributed evenly, as respects their vital conditions, and thus effect a diminution of the magnitude of error due to individual cell differences. This procedure, in fact, gave more comparable results.

TABLE III.

Tube No.	First count.	Second count (after 18 hrs.).	Average.
	cells	*cells*	*cells*
1	21	23	22
2	132	92	112
3	214	198	206
4	342	308	325
5	602	684	643

By the counting chamber method, and using the same suspension already used with the droplet method, the results given in Table III were secured.

The largest variation in the series of countings on the same suspension was in Tube 5 (602 to 684—difference 82, about 13 per cent variation between the first and second counting).

It seems, therefore, that a more even and reliable result can be obtained by the use of this procedure than with the droplet method devised by Williams, even though the droplets were made by the platinum needle instead of a pen.

We have tested the effect of increasing the quantity of the first aqueous extraction of wheat germ on the growth of yeast. The uniformly beneficial effects were suggestive that the high content of water-soluble B in the extract exerted a stimulating effect on the growth of the yeast.

The results of testing the second and third extracts prepared from the residue of the first extraction when tested for their effect on the growth of yeast are given in Tables IV and V.

TABLE IV.

Test with Second Extract.

	No. of cells.		Average after 12 hrs. incubation.
Control solution	21		21
" " + 0.1 cc	132	92	112
" " + 0.2 "	214	198	206
" " + 0.4 "	342	308	325
" " + 1.0 "	602	684	643

TABLE V.

Test with Third Extract.

	No. of cells.		Average after 24 hrs. incubation.
Control solution	150	189	174.5
" " + 1.0 cc	378	366	372
" " + 2.0 "	421	480	450.5
" " + 3.0 "	511	554	532.5
" " + 1.0 " first extract	1,068	1,240	1,154
" " + 1.0 " second "	649	633	641

The results show clearly that there is a stimulating effect of the extracts on the rate of multiplication of yeast and that in general the greater the amount of extract added the more pronounced the acceleration.

Alcoholic extracts of wheat germ were also prepared in which various strengths of alcohol were used, varying from 40 to 75 per cent. In all cases the germ was boiled for 10 minutes in alcohol and the solution filtered through paper or an alundum filter, then evaporated on a water bath at low temperature with a fan blowing on the surface. In all cases it was observed that the growth of yeast was markedly stimulated by the addition of an alcoholic extract of wheat germ to the control solution.

In some experiments we have used wheat germ extract treated with sodium bicarbonate and autoclaved, in order to destroy as much as possible of the antineuritic substance. Before addition of such extracts to the nutrient medium the sodium bicarbonate was neutralized with hydrochloric acid.

Extract of fresh beef made by boiling the latter with water was also employed. The beef muscle was treated with 2 per cent of $NaHCO_3$, moistened and allowed to stand for 3 hours, then autoclaved 1 hour at 15 pounds pressure. Extract of rolled oats was also used. The oats were treated with 2 per cent of $NaHCO_3$ and autoclaved at 15 pounds pressure as described for the meat muscle. The oats so treated were fed to young rats in a diet consisting of rolled oats 40.0, gelatin 5.0, casein 5.0, NaCl 1.0, butter fat 3.0, $CaCO_3$ 1.5 per cent. The animals failed to grow. After 41 days 3 per cent of untreated wheat germ was added to the diet to furnish the antineuritic substance. The animals responded at once with rapid growth. 40 per cent of untreated oats furnishes an abundance of antineuritic substance, water-soluble B, for growth. The treatment of the oats with alkali had, therefore, destroyed its content of this substance.

The series has been extended by the employment of meat extract prepared by precipitation with barium, the addition of barium being stopped while the solution was still distinctly acid. After the removal of the barium sulfate the remaining acidity was neutralized by ammonium hydroxide.

All extracts employed in our experiments were carefully sterilized by boiling and kept in an ice box until used. Observations were made to establish that there was no contamination of these extracts with bacteria.

Table VI illustrates the effect of 40 per cent alcoholic extract of wheat germ added to the control solution on the rate of development of yeast cells. Equal amounts of successive extracts were employed.

Table VII illustrates the effect of adding 1 cc. of each of a series of eight successive extracts of wheat germ, using 40 per cent alcohol as a solvent.

Table VIII illustrates clearly the progressively increasing benefit to the yeast by adding increasing amounts of 75 per cent alcoholic extract of wheat germ.

TABLE VI.

	No. of cells after 18 hrs.		Average.
5 cc. of control solution	147	133	140
5 " " " " + 1 cc. first extract.	811	798	804.5
5 " " " " + 1 " second "	507	509	508
5 " " " " + 1 " third "	392	416	404
5 " " " " + 1 " fourth "	430	399	414.5

TABLE VII.

	No. of cells after 20 hrs.			Average.
5 cc. control solution	570	433	480	494.3
5 " " " + 1 cc. first extract	1,560	1,720		1,640
5 " " " + 1 " second "	1,610	1,720		1,665
5 " " " + 1 " third "	890	810		850
5 " " " + 1 " fourth "	760	670		715
5 " " " + 1 " fifth "	590	690		640
5 " " " + 1 " sixth "	820	680	780	760
5 " " " + 1 " seventh "	760	560		660
5 " " " + 1 " eighth "	500	600		550

TABLE VIII.

	No. of cells after 20 hrs. incubation per 400 squares.		Average.
5 cc. control solution + 1.0 cc. water	499	483	491
5 " " " + 0.9 " " + 0.1 cc. first extract	1,000	870	935
5 cc. control solution + 0.8 cc. water + 0.2 cc. first extract	1,360	1,390	1,375
5 cc. control solution + 0.6 cc. water + 0.4 cc. first extract	1,610	1,740	1,675
5 cc. control solution + 1.0 cc. water + 1.0 cc. first extract	2,180	2,540	2,360

Tables IX, X, and XI illustrate the effect of adding to the control solution aqueous or alcoholic (40 per cent alcohol) extracts of wheat germ. In these experiments the wheat germ had

TABLE IX.

	No. of cells after 18 hrs. per 400 squares.		Average.
5 cc. control solution	770	580	675
5 " " " + 1 cc. autoclaved wheat germ extract	2,460	1,860	2,160
5 cc. control solution + 2 cc. autoclaved wheat germ extract	2,650	3,530	3,090
5 cc. control solution + 1 cc. alcoholic extract of wheat germ	2,520	2,420	2,470
5 cc. control solution + 2 cc. alcoholic extract of wheat germ	2,530	2,320	2,425

TABLE X.

	No. of cells after 18 hrs. per 400 squares.		Average.
5 cc. control solution	160	210	185
5 " " " + 1.5 cc. water + 0.5 cc. 75 per cent alcoholic extract	1,340	1,210	1,275
5 cc. control solution + 1.0 cc. water + 1.0 cc. 75 per cent alcoholic extract	2,060	2,030	2,045
5 cc. control solution + 1.0 cc. water + 0.5 cc. 75 per cent alcoholic extract + 1.0 cc. autoclaved wheat germ	1,920	1,480	1,700
5 cc. control solution + 1 cc. autoclaved wheat germ + 1 cc. 75 per cent alcoholic extract	2,790	3,920	3,355
5 cc. control solution + 1 cc. autoclaved wheat germ + 1 cc. water	2,190	2,120	2,155

TABLE XI.

	No. of cells after 20 hrs. per 400 squares.		Average.
5 cc. control solution + 2 cc. water	100	120	110
5 " " " + 1 " " + 1 cc. extract autoclaved wheat germ	2,440	2,320	2,380
5 cc. control solution + 2 cc. extract autoclaved wheat germ	2,730	2,630	2,680
5 cc. control solution + 1 cc. water + 1 cc. 40 per cent alcoholic extract wheat germ	2,040	2,320	2,180
5 cc. control solution + 2 cc. 40 per cent alcoholic extract wheat germ	2,190	1,670	1,930

been treated with 2 or 4 per cent sodium bicarbonate, allowed to stand in a moist condition for 3 hours or longer, in order that the alkali might penetrate the material. The wheat germ was then autoclaved at 15 pounds pressure for 3 hours, the acid neutralized, and the material dried in a current of warm air.[2]

TABLE XII.

	24 hrs.		48 hrs.	
	Counts.	Average.	Counts.	Average.
1. 10 cc. control solution.	300 310	305	2,350 2,500	2,425
2. 10 " " " + 1 cc. 10 per cent glucose.	390 330	360	5,020 4,940	4,980
3. 10 cc. control solution + 2 cc. 10 per cent glucose.	360 380	370	8,300 7,650	7,975
4. 10 cc. control solution + 1 cc. autoclaved wheat germ extract.	1,540 1,350	1,445	2,880 3,060	2,970
5. 10 cc. control solution + 1 cc. autoclaved wheat germ extract + 1 cc. 10 per cent glucose.	1,400 1,390	1,395	3,780 4,020	3,900
6. 10 cc. control solution + 1 cc. 75 per cent alcoholic wheat germ extract.	1,650 1,810	1,730	5,340 5,450	5,395
7. 10 cc. control solution + 1 cc. 75 per cent alcoholic wheat germ extract + 1 cc. 10 per cent glucose.	1,570 1,540	1,555	5,400 5,760	5,580

It is easily apparent that the addition of these extracts exerted a profound stimulating effect on the growth of the yeast, but that the extent of acceleration of the growth of yeast is not proportional to the amount of extract added.

---

[2] This germ was used in a feeding experiment with rats to show whether it still contained any appreciable amount of water-soluble B. The ration consisted of casein 18, treated wheat germ 15, butter fat 5, salt mixture (No. 185) 3.7, dextrin 58.3 per cent. The animals failed to grow. In a subsequent period rapid growth took place when 3 per cent of untreated germ was included in the diet. All the antineuritic substance had been practically destroyed by heating with alkali.

Table XII illustrates the effect on the growth of yeast of adding to the control solution varying amounts of glucose and of glucose together with extracts of wheat germ.

It will be observed that Nos. 2 and 3 show a surprising acceleration due to the addition of double the amount of glucose. 1 cc. of 10 per cent glucose solution added to 10 cc. of control solution produced 4,980 yeast cells after 48 hours. 2 cc. of the glucose solution added to 10 cc. of control solution led to the development of 7,975 cells in the same interval. Comparing Nos. 4 and 5, the addition of 1 cc. of autoclaved wheat germ extract produced 2,970 cells in 48 hours, whereas the same medium with 1 cc. of 10 per cent solution produced 3,900 cells. That results are not always consistent is shown by comparison of Nos. 6 and 7, in which there was a marked effect from the addition of 1 cc. of 75 per cent alcoholic extract of wheat germ, and very slight further stimulation by the addition of 1 cc. of 10 per cent glucose.

Table XIII illustrates further the lack of uniformity in the development of yeast under different conditions.

Comparing Nos. 2 and 3, greater growth was secured by adding to 10 cc. of the control solution 1 cc. of 10 per cent glucose solution than was obtained by the addition of 0.1 cc. of 75 per cent alcoholic extract of wheat germ without additional glucose. In order to test the possibility of securing comparable development of yeast when the cultural conditions for every cell were as nearly as possible identical the following procedure was tried.

A drop of yeast suspension was placed on a clean cover-slip, and the number of yeast cells contained in it carefully determined by counting. The yeast cells were then immediately washed into a sterile test-tube by means of control solution, and the amount of control solution was then adjusted so that 0.1 cc. of nutrient solution was available for each yeast cell present. The following five experiments illustrate the results of this procedure. An examination of Table XIV shows that in the first test-tube there were 21 yeast cells, each with 0.1 cc. of nutrient medium. After 12 hours the number was increased to 39, an approximate multiplication of the original number by 2. In the second tube 29 cells were present, and in 12 hours this had multiplied the original number by 16. In the third tube 31 cells were present, and after 12 hours this had multiplied the original number by 19

In the fourth tube 34 cells were present, and after 12 hours this had multiplied 21½ times the original number. In the fifth tube 43 cells were present and in 12 hours the original number was multiplied by 10. The cause of these irregular results is unquestionably to be attributed to the introduction of injured cells or cells of low reproductive power into the test specimens.

TABLE XIII.

	18 hrs.		52 hrs.	
	Counts.	Average.	Counts.	Average.
1. 10 cc. control solution.	350 290	320	4,680 4,100	4,390
2. 10 " " " + 1.0 cc. 10 per cent glucose.	420 380	400	4,660 4,620	4,640
3. 10 cc. control solution + 0.1 cc. 75 per cent alcoholic extract wheat germ.	620 670	645	3,600 3,760	3,680
4. 10 cc. control solution + 0.1 cc. 75 per cent alcoholic extract wheat germ + 1.0 cc. 10 per cent glucose.	910 980	945	6,260 5,320	5,790
5. 10 cc. control solution + 0.5 cc. 75 per cent alcoholic extract wheat germ.	940 1,300	1,120	4,240 4,400	4,320
6. 10 cc. control solution + 0.5 cc. 75 per cent alcoholic extract wheat germ + 1 cc. 10 per cent glucose.	930 890	910	4,340 4,452	4,396
7. 10 cc. control solution + 1 cc. 75 per cent alcoholic extract wheat germ.	1,250 1,800	1,525	4,420 4,590	4,505
8. 10 cc. control solution + 1 cc. 75 per cent alcoholic extract wheat germ + 1 cc. 10 per cent glucose.	1,130 1,240	1,185	4,380 4,190	4,285

We have sought to test the value of a procedure involving the addition of 1 drop of opalescent suspension of yeast cells in water to several test-tubes, each containing 10 cc. of nutrient solution, and then counting these cells in the Levy chamber before incubation.

Table XV illustrates one set of experiments in which fairly close agreement was obtained by this procedure, but the agreement is presumably due to the good fortune of selecting a series of cells all or nearly all of which were capable of development.

In Table XVI are recorded the results of a series of trials designed to determine the effect of various extracts on the rate of development of yeast. The volume of solution was kept constant by the addition of glucose solution or water. The materials from which the extracts were prepared in these experiments had been subjected to heat under pressure in the presence of sodium bicarbonate as described in the methods of preparation of materials. In all cases these preparations had been tested with rats and found to be at least practically free from the dietary factor water-soluble B. Notwithstanding the fact that they con-

TABLE XIV.

Tube No.	Original count.	Control solution.	Count after 12 hrs.	Average.
	cells	*cc.*	*cells*	*cells*
1	21	2.1	43 35	39
2	29	2.9	464 461	462.5
3	31	3.1	594 586	590
4	34	3.4	767 698	732.5
5	43	4.3	422 454	438

TABLE XV.

Tube No.	Count per 400 squares.	Count after 22 hrs.		Average.
	cells	*cells*		*cells*
1	3	186	198	192
2	4	174	195	184.5
3	3.5	169	153	161

tained so little of this substance as to be undemonstrable by experiments on the rat the extracts were still very effective in stimulating the growth of yeast. These results suggest that although the addition of the antineuritic substance in minute amounts may have influenced the growth of yeast in these experi-

TABLE XVI.

	No. of cells after 18 hrs.		Average.
1. 9 cc. control solution. 1 " 10 per cent glucose solution. 1 " H_2O.	390	350	370
2. 8 " control solution. 2 " glucose " 1 " H_2O.	310	380	345
3. 8 " control solution. 2 " glucose " 1 " eighth extract wheat germ.	320	340	330
8 " control solution. 2 " dextrose " 1 " autoclaved wheat extract (first).	1,750	1,610	1,680
5. 8 " control solution. 2 " glucose " 0.5 " first extract wheat germ. 0.5 " H_2O.	1,880	1,750	1,815
6. 8 " control solution. 2 " glucose " 1 " first extract wheat germ.	1,960	1,990	1,975
7. 8 " control solution. 2 " glucose " 1 " rolled oats extract (made from rolled oats autoclaved with 2 per cent $NaHCO_3$).	1,980	2,010	1,995
8. 8 cc. control solution. 2 " glucose " 1 " beef extract (from beef steak autoclaved with 2 per cent $NaHCO_3$).	1,700	1,760	1,730

TABLE XVII.

	No. of cells after 20 hrs.		No. of cells after 43 hrs.		Gas.
	Counts.	Average.	Counts.	Average.	
					cc.
1. 9 cc. control solution. 1 " 10 per cent glucose solution. 1 " H_2O.	156 137	146.5	2,510 2,880	2,695	2.5
2. 8 " control solution. 2 " glucose " 1 " H_2O.	143 139	141	2,300 2,180	2,240	2.5
3. 8 " control solution. 2 " glucose " 1 " eighth extract wheat germ.	133 128	130.5	1,740 2,010	1,875	0.5
4. 8 " control solution. 2 " glucose " 1 " autoclaved wheat extract (first).	732	732	4,180 4,230	4,205	10
5. 8 " control solution. 2 " glucose " 0.1 " first extract wheat germ.	493 535	514	3,900 4,780	4,340	4
6. 8 " control solution. 2 " glucose " 0.5 " first extract wheat germ (alcohol). 0.5 " H_2O.	675	675	5,020 5,210	5,115	5
7. 8 " control solution. 2 " glucose " 1 " first extract wheat germ (alcohol).	1,340	1,340	4,740 4,100	4,420	8
8. 8 " control solution. 2 " glucose " 1 " fifth extract wheat germ (alcohol).	170 143	156.5	4,830 5,140	4,985	8
9. 8 " control solution. 2 " glucose " 1 " extract rolled oats (fresh oats).	636 752	694	3,680 4,310	3,995	8
10. 8 " control solution. 2 " glucose " 1 " steak extract (alkalinized steak).	1,027	1,027	4,560 4,940	4,750	10
11. 8 " control solution. 2 " glucose " 1 " third extract wheat germ (alcohol).	880	880	2,230 2,340	2,285	3

ments, this factor is so small as to make it practically certain that other added substances, as glucose and amino-acids, also served to influence the results in a marked way.

Table XVII illustrates the results obtained in a series of experiments in which it was sought to estimate by the gas produced by the yeast culture the extent of growth of the yeast in several types of modified nutrient medium. These results are so variable, when we compare the gas production with the number of yeast cells generated during the period of incubation, that the measurement of the amount of gas produced does not give promise of value in this line of work.

TABLE XVIII.

	No. of cells after 20 hrs.		Average.
1. 1 cc. control solution.	1,020	1,230	1,125
2. 1 " " " 1 " autoclaved wheat extract.	1,980	2,080	2,030
3. 10 " control solution. 0.5 " digested meat (prepared from fresh steak not autoclaved with alkali).	1,820	1,970	1,895
4. 10 cc. control solution. 0.5 " digested meat extract (prepared from fresh steak not autoclaved with alkali).	2,048	2,200	2,124
5. 10 cc. control solution. 1 " digested meat extract (prepared from fresh steak not autoclaved with alkali).	2,412	1,940	2,176
6. 10 cc. control solution. 1 " first extract wheat germ.	2,020	2,380	2,200
7. 10 " control solution. 0.5 " first extract wheat germ.	1,680	1,880	1,780

In Table XVIII is presented a series of observations on the effect of adding an amino-acid mixture obtained by the hydrolysis of beef muscle with sulfuric acid.

So far as our knowledge permits us to judge, these digested meat extracts could serve only as a source of amino-acids, since the treatment was sufficient to reduce the proteins practically to the amino-acid state, and, since muscle tissue is, even in the fresh condition, very poor in the antineuritic factor (water-soluble B), the same meat after hydrolysis with strong sulfuric acid would scarcely contain a trace of it. Yet these meat digests exerted a profound influence on the rate of development of yeast, approximately doubling the number of cells which were counted after 20 hours incubation in Experiments 6 and 7, Table XVIII. It is again apparent that doubling the amount of the antineuritic substance derived from wheat germ extract does not exert a proportional stimulating effect on the growth of yeast.

The series of observations which we have presented above seem to us to form a conclusive demonstration that the use of yeast as a test organism for determining the presence or absence of the antineuritic dietary factor is complicated by so many disturbing factors as to make it of little if any value. Whenever extracts of natural foods are to be tested it is inevitable that food substances of one kind or another which greatly stimulate the growth of yeast must be added simultaneously with the unidentified dietary essential for which the test was designed. Glucose and amino-acids effect such stimulation

We do not desire to draw the deduction from the data here presented that the antineuritic substance is not necessary for the development of yeast, or that it does not stimulate the growth of yeast, but the several modifications of the procedure originally described for the utilization of yeasts as a test for the antineuritic substance point so definitely to several difficulties in the way of making this a satisfactory test that it has seemed to us desirable to present the results for the benefit of those who may be interested in this line of work.

HEMATO-RESPIRATORY FUNCTIONS.

VI. THE ALTERATION OF THE CO_2 RATIO ($H_2CO_3 : NaHCO_3$) IN THE BLOOD DURING ELEVATION OF BODY TEMPERATURE.

BY HOWARD W. HAGGARD.

(From the Physiological Laboratory of Yale University School of Medicine, New Haven.)

(Received for publication, August 7, 1920.)

Increase in temperature lowers the solubility coefficient of CO_2 in blood. It also increases the tension of water vapor in the lungs, and this tends to reduce the partial pressure of CO_2. Both conditions act, therefore, to reduce the dissolved CO_2, and presumably the H_2CO_3, in the arterial blood. On merely physicochemical grounds we should, therefore, expect that increase in body temperature, whether from fever or from extrinsic causes, *e.g.* hot packs or baths, would depress respiration to a degree sufficient to allow the alveolar CO_2 tension to rise and thus counteract the reduction in the solubility of CO_2.

This, however, is not the case. On the contrary, rise of body temperature is accompanied by an augmentation of respiration and a fall in the alveolar CO_2 tension in excess of the decrease produced by the higher partial pressure of water vapor in the lungs. These physiological relations are to be seen in the following experiments. All of those performed *in vivo* were upon myself.

Experiment 1.—Subject, normal, human, adult, male. Body temperature was increased by immersion up to the chin in water at 43°C. for 20 minutes. Oral temperatures taken. Alveolar air obtained by Henderson sampler (1).

	Oral temperature.	Alveolar CO_2.
	°C.	*mm.*
Normal resting	37	39.9
After 20 min. immersion	40	35.0
Difference	+3	−4.9

The increase of water vapor in the lungs from 46.65 mm. at 37°C. to 54.87 mm. at 40°C., unless compensated in some way, would result in a fall of 0.5 mm. in CO_2 alveolar tension, or 0.07 per cent. The experimental findings here indicate a decrease in the dry gas percentage from the normal of 5.6 per cent to 4.9 per cent at 40°C., a fall of 0.7 per cent or 4.9 mm. CO_2.

Overbreathing of the amount thus indicated is not sufficient to effect in the brief period of such an experiment any considerable lowering of the available blood alkali, as expressed in the dissociation curve, although it would of course induce an immediate slight decrease of the alkali in use, as expressed by a shifting of the arterial point along the curve to the left. That the blood alkali is not sensibly lowered is to be seen in the following experiment.

Experiment 2.—General procedure as in previous protocol. Blood samples were drawn from the median basilic vein before and at the end of the period of immersion and were equilibrated at 37°C. with CO_2 at 40 mm. tension.

20 minutes immersion at 43.2°C.

	Oral temperature.	Alveolar CO_2.	CO_2 combined by blood at 40 mm. tension, 37°C.
	°C.	*mm.*	*vol. per cent*
Normal resting	37.0	39.2	47
After 20 min. immersion	40.8	33.9	47
Difference	+3.8	−5.3	0

The fall in alveolar CO_2 tension due to water vapor in this experiment would amount to 0.6 mm. An actual fall of 5.3 mm. occurred. This represents a decrease in dry CO_2 from the normal of 5.5 per cent to 4.8 per cent.

As indicated by the CO_2-combining power at 40 mm. CO_2 tension, 37°C., there was no absolute decrease in available blood alkali.

Experiment 3.—Normal blood of the same person equilibrated at 35° and at 40°C. with 39.5 and 38.7 mm. CO_2, respectively. Analysis and calculation showed the CO_2 content, dissolved and combined CO_2, and CO_2 ratio as follows:

	CO_2 content.	CO_2 dissolved.	CO_2 combined.	CO_2 ratio.
	vol. per cent	*vol. per cent*	*vol. per cent*	*per cent*
At 35°C. and 39.5 mm. CO_2........	48	2.90	45.10	6.4
" 40 " " 38.7 " CO_2........	47	2.58	44.42	5.8

Increase in body temperature results in a considerable fall in the alveolar CO_2, and, therefore, probably in the CO_2 dissolved in the arterial blood. This decrease, as is shown in Experiment 2, is not compensated by a fall in the available alkali of the blood, for this remained constant. Unless some alteration in the CO_2-carrying power of the blood occurs in response to change in temperature, a displacement to some new value in the balance between the free and combined CO_2, the CO_2 ratio, of the arterial blood must occur and, presumably, a change in C_H result.

That such a change does occur in the CO_2-combining power of the blood but that it is of a magnitude insufficient to compensate the fall in dissolved CO_2, is shown in the following section.

The Influence of Temperature Upon the CO_2-Carrying Power of Blood in Vitro.

Christiansen, Douglas, and Haldane (2) cite one equilibration to illustrate the effect of temperature upon the CO_2-combining power of defibrinated blood. Van Slyke, Stillman, and Cullen (3) have shown the extent of this change in plasma. The quantitative relations for whole blood, however, have not been made clear. That temperature does influence to some extent the equilibrium between carbon dioxide and the buffer substances of the blood, independent of the effect on carbon dioxide solubility, has been shown by L. J. Henderson (4).

The greatest determining factor, however, in placing the level of alkali in use, must be the dissolved CO_2 in relation to the dissociation curve. In order to test quantitatively the degree of response of alkali to the dissolved CO_2, dissociation curves were plotted from normal dog blood at temperatures of 17°C. or 20° and 40°C. The curves so obtained were then compared at those points which represented like amounts of CO_2 in solution. Coincidence at these points would indicate a complete response of the alkali to the dissolved CO_2 independent of other effects of temperature.

Normal blood from four dogs ((*a*), (*b*), (*c*), and (*d*)) was oxalated and then equilibrated at 40°C. with CO_2 at three tensions. A second portion of the same blood was then equilibrated at 17°C., or at 20°C., with CO_2 at three tensions.

The CO_2 retained by the blood after each equilibration minus the CO_2 in solution is given in Table I.

TABLE I.

Blood.	Temperature 17°C.			Temperature 40°C.		
	Equilibrating tension CO_2.	Combined CO_2.	Dissolved CO_2.	Equilibrating tension CO_2.	Combined CO_2.	Dissolved CO_2.
	mm.	*vol. per cent*	*vol. per cent*	*mm.*	*vol. per cent*	*vol. per cent*
(*a*)	22.4	39.35	2.65	21.2	28.61	1.39
	41.0	49.15	4.85	38.8	35.45	2.55
	74.6	63.18	8.82	70.5	49.37	4.63
(*b*)	22.4	29.35	2.65	21.2	18.61	1.39
	41.0	35.15	4.85	38.8	28.45	2.55
	74.6	45.12	8.82	70.5	37.37	4.63
	Temperature 20°C.			Temperature 40°C.		
(*c*)	16.1	36.12	1.80	15.2	24.00	1.00
	40.8	50.23	4.73	38.8	39.45	2.55
	81.6	60.45	9.55	77.6	49.88	5.12
(*d*)	16.1	30.12	1.80	15.2	22.00	1.00
	40.8	42.23	4.73	38.8	34.45	2.55
	81.6	50.45	9.55	77.6	43.88	5.12

The curves from Bloods (*a*) and (*b*) are plotted in Fig. 1. The close coincidence of the curves at 17° and 40°C. at those points which represent like amounts of CO_2 in solution is apparent. The curves at 40°C. tend to run slightly lower than those at 17°C., but hardly in excess of the error of the method.

Rise in body temperature from all the aspects approached in this paper tends to displace to a new value the ratio of H_2CO_3: $NaHCO_3$ in the blood.

In order adequately to compensate the fall in dissolved CO_2 the dissociation curve would need to be of such a shape that all values of alkali maintain a constant ratio with the dissolved CO_2. The curves are entirely too flat to allow this, and the C_H is therefore decreased.

These observations tend to confirm the supposition that under increase in body temperature the CO_2 ratio is reduced by physiological, even more than by mechanical, factors.

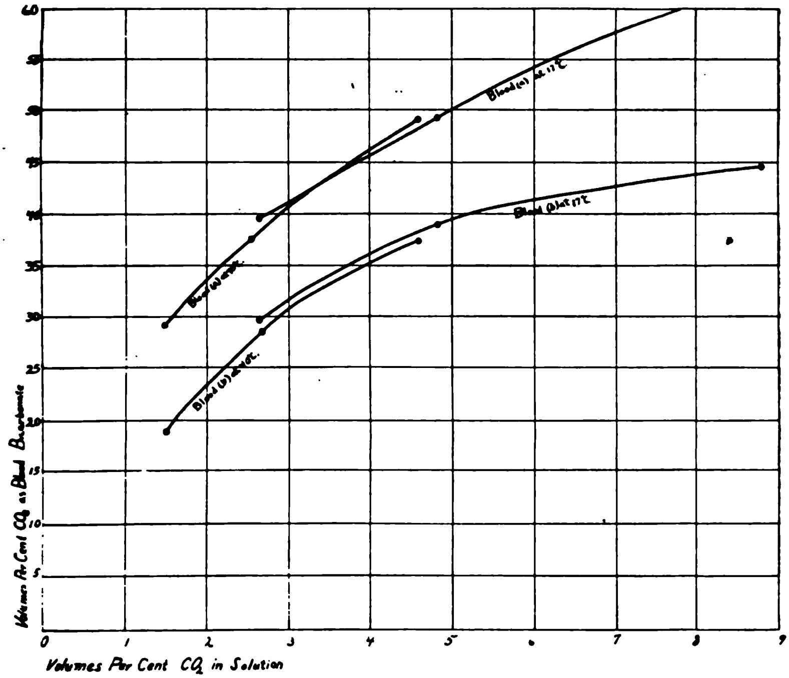


FIG. 1.

SUMMARY.

Rise in body temperature lowers the alveolar CO_2 tension and reduces the amount of CO_2 in solution in blood.

No compensatory changes in the CO_2-combining power of the blood occurs.

The alkali in use is not lowered in proportion to the decrease in dissolved CO_2.

The CO_2 ratio is therefore reduced and, unless there is some alteration in the dissociation constants of the blood, the C_H is presumably lowered (5).

In vitro temperature affects the dissociation curve of blood solely by altering the solubility of CO_2 and concentration of H_2CO_3. The concentration of H_2CO_3 which produces a certain amount of $NaHCO_3$ at 17° or 20°C. produces the same amount at 40°.

BIBLIOGRAPHY.

1. Henderson, Y., and Morriss, W. H., *J. Biol. Chem.*, 1917, xxxi, 217.
2. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
3. Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 401.
4. Henderson, L. J., *Am. J. Physiol.*, 1905–06, xv, 257.
5. Henderson, L. J., *Ergebn. Physiol.*, 1909, viii, 254.

THE NUTRITIVE PROPERTIES OF MILK, WITH SPECIAL REFERENCE TO REPRODUCTION IN THE ALBINO RAT.

By H. A. MATTILL and RUTH E. CONKLIN.

(*From the Department of Physiology, University of Rochester, Rochester, N. Y.*)

(Received for publication, July 28, 1920.)

The nutritive efficiency of the various known and unknown constituents of milk has been tested out repeatedly on different types of experimental animals, particularly on the rat, usually by adding milk or one of its constituents to a basal ration deprived in whole or in part of the substance in question, less often by using milk as a basal ration and superimposing other substances upon it. It is unnecessary to review extensively the compendious literature on this subject since recent papers on particular phases contain abundant references.

The relative value of the milk proteins is of a high order (1–3) although under certain conditions lactalbumin, supposedly the most complete of all the milk proteins, has appeared not to be so (4, 5). Milk sugar, long known to possess desirable characteristics not found in the other disaccharides, has been shown to be the carrier of water-soluble vitamine unless carefully purified (6, 7) and a complementary relation of lactose to lactalbumin has been suggested (5). Milk fat occupies its unique position by virtue of possessing fat-soluble vitamine (8, 9), whose nature and functions in the organism are still an unsolved problem (10), and whose origin and distribution in nature continue to occupy the interest of a number of investigators. As great a mystery surrounds the nature and function of the water-soluble vitamine of milk, first brought to light in the work of Hopkins (11) and used in the form of "protein-free milk" by Osborne and Mendel in a long series of investigations. The question as to its identity with the water-soluble vitamine from other sources, notably yeast, was raised several years ago (12, 13) and has been renewed in a recent critical paper (14); evidence has also been presented to show that the water-soluble vitamine of milk may indeed be unique and different from that present in other foodstuffs (15). The antiscorbutic value of milk has been demonstrated (16) although this may sometimes be inconsiderable. The inorganic salts of milk are successfully used as a source of mineral requirement of the rat (17).

Incidental to and growing out of the above have been investigations as to the effect of heat, storage, and other factors on the various constituents, notably the proteins (13, 18), the fat-soluble (19–21), and antiscorbutic vitamines (16, 22). A quantitative measure of these effects is necessary if we are to know the absolute requirements of the experimental animal and the adequacy of milk to meet them; for neither of these quantities is there as yet an absolute measure, at least not as regards the vitamines. Since it is probable that the amount of vitamines present in milk is dependent among other things on the amounts present in the feed of the animal giving the milk (23) and this in turn upon seasonal variations (22) and stage of maturity of the plant (24), we are obviously still far from a definite answer to these questions.

As the first and sole nourishment of the young animal milk is considered a perfect food and certainly no other single product of animal or vegetable life used for food so fully provides for the nutritive needs of growing (and adult) animals as does milk. Indeed many of these requirements were not recognized until it was discovered that the addition of milk or of its derivatives could correct faulty diet. After a young animal is weaned its nutritive demands are met by a variety of food materials among which, except in the case of man and his domestic animals, milk is not found. The new foods to which an animal is introduced after weaning are generally characterized by a lower protein content, a wider variety of mineral sources, and by the addition of a new type of material, roughage. The interesting question presents itself as to whether milk could continue to be an adequate food for the whole span of life or whether it is lacking in some factor which is necessary for the normal performance of physiological function and for the fulfillment of the normal expectancy; whether it possesses in too great abundance a factor which would prevent normal behavior either because of a consequent improper balance among the ingredients or through bringing about early cessation of function of a gland or organ. In the answer to this question might also be found some indication of the variation in food requirements during the different periods or cycles into which the life of an animal may be divided; any deterioration which milk might suffer in the process of evaporation or desiccation, if not made obvious during brief periods of experimentation, might thus be brought to light, under prolonged scrutiny.

Practically all our information on milk concerns itself with cow's milk and its derivatives; excepting the experiments on scurvy, the animals whose behavior forms the basis of the conclusions drawn are mostly rats. In her comprehensive book on milk (25) Lane-Claypon devotes several chapters to the nutritive value of milk and the data then available from experiments on various species of animals with milks from the same and other species. All these investigations were of relatively short duration, in very few of them was milk the sole food, and the conception of vitamines, then in its earliest infancy, had not yet modified the entire aspect of nutritive values. The difficulties of such animal experimentation are also pointed out with particular emphasis on the dissimilarity of milks, the complete absence of information as to the composition of milk of some species, differences in the condition of young animals at birth, different rates of growth in different species, a varying rate of growth in the same species with the consequent changing demands of the organism, and the great factor of variability.

The present investigation of which this is a preliminary report was begun to secure information on the food value of cow's milk and its products from the usual type of laboratory animals, several of which have thus far been under observation. This paper includes only the first findings obtained from rats.

EXPERIMENTAL.

Young rats from our stock colony were placed on the experimental rations at the time of weaning, two in a cage and usually mated from different litters. Their average age at this time was 25 days, and only three of the series were as old as 35 days. Their average weight was about 30 gm., and a few from small litters weighed as much as 40 or 50 gm. The fresh milk used was Grade B raw and was placed in the cages each morning in amounts as indicated by the previous day's consumption. Next morning the amount remaining, if any, was measured back. Usually this was very little and it was found sweet to the smell as often as sour, showing that contamination, while probably always present, was not extensive. The dry milk food[1] was renewed every 2nd or 3rd day, and while scattering was common it was possible to secure a fair idea of food consumption especially when the amounts supplied closely approximated the requirement. Water was always available in inverted tubes. The animals were weighed twice a week until fairly grown, thereafter weekly.

---

[1] Ambrosia and Klim were obtained from the Mammala Corporation and the Merrell-Soule Company, respectively.

In Charts 1 and 2 are found the growth curves of males and females, respectively, on a diet of fresh cow's milk with the various additions that were later made. It was somewhat surprising to find rats making such satisfactory initial growth on cow's milk considering the differences in composition and concentration between cow's and rat's milk (26). The later growth, however, was unsatisfactory both in males and females. Between the 50th and 100th day of life, especially in females, there was a decided retardation of growth, sometimes a considerable loss in weight, after which growth might continue but at a slower rate and on a much lower level. The animals on fresh milk failed to reproduce although there was satisfactory reproduction in animals from the same litters placed on stock rat food. The records of milk consumption are summarized in Tables I and II. This summary does not include all the animals because through regrouping and remating the record of a given pair was necessarily closed. Only those figures are given, therefore, which cover a substantially unbroken period, beginning at the time of weaning when the animals were placed on the experimental ration. In general the food consumption runs somewhat parallel with the rate of growth and a comparison of the fresh and dry milk figures shows that the amount of liquid which had to be ingested in fresh milk tended to diminish the consumption of milk solids in that form. Most of the fresh milk rats did not always consume milk to their capacity judging from the larger amounts of which they disposed in some periods. Perhaps the factors of humidity and temperature had some effect but of these we have no record. For the peculiar behavior of Rats 3025, 3026, 3015, and 3016 when they were regrouped and mated there seems no explanation. Food consumption fell appreciably after the mating, the rate of growth of the females was permanently slower, and the males, after a decline in weight for several weeks, renewed their growth somewhat but on a lower level. Of particular interest in this connection are the results recently

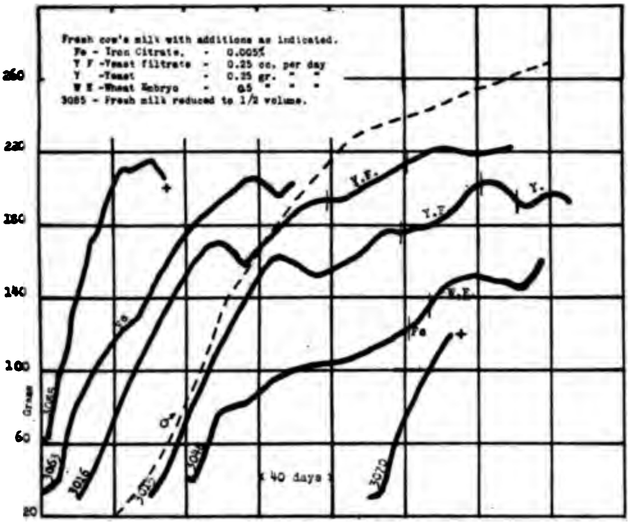


CHART 1.

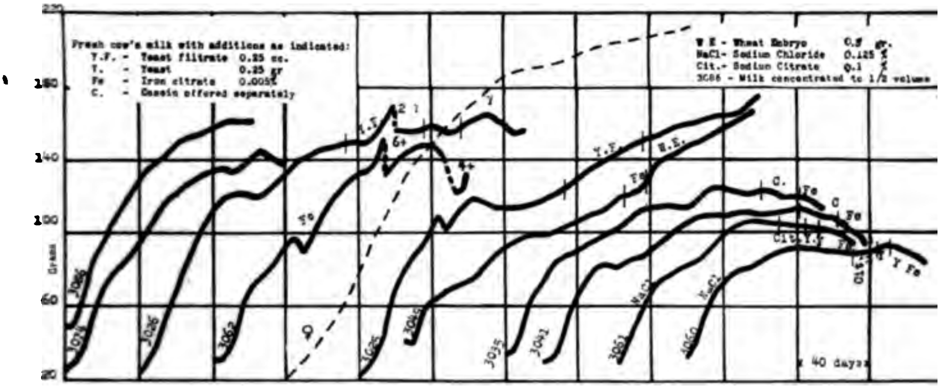


CHART 2.

TABLE I.

Food Intake of Rats on Fresh Milk.

The food intake is given in terms of solids (one-eighth of the volume of milk consumed) and the figures represent the average daily ingestion of two rats over 10 day periods beginning at the time of weaning. Unless otherwise indicated the animals were mated.

Nos. 3060, 3061.	Nos. 3062, 3063.	Nos. 3048, 3049.	Nos. 3025♀, 3026♀.	Nos. 3015♂, 3016♂.	Nos. 3035♀, 3041♀.	Nos. 3027♀, 3028♀.*	Nos. 3085, 3086.†
7.0	6.3	5.6	6.8	6.4	6.3	5.0	9.4
8.6	10.7	8.1	10.3	10.9	9.3	9.0	14.2
7.4	11.1	7.7	12.6	13.2	10.0	9.0	17.9
7.7	10.0	8.0	12.6	12.9	8.3	10.5	16.9
8.3	10.4	8.4		13.6	8.1	9.8	16.8
7.4	11.3	8.7		14.7	8.7	9.5	14.4
			Mated.				
			Nos. 3025, 3016.	Nos. 3015, 3026.			
7.4	13.2	8.2	10.2	12.1	8.2	11.8	13.0
6.1	11.8	7.7	10.2	10.2	8.0	13.2	
5.9	13.4	6.9	10.6	10.5		13.7	
5.7	13.0	7.9	12.0	11.7		13.2	
5.7	12.7	7.9	12.8	12.2		12.6	
5.8	12.0	8.3	11.2	11.4		13.8	
5.0	8.2	8.3	10.5	11.0		14.1	
	14.0	10.5	11.8	13.3		14.1	
		10.4	11.3	10.9		14.4	
		8.8	11.8	10.8		13.5	
		9.5	12.0	12.5		13.3	
		9.7	11.1	12.3		13.5	
			12.2	11.8		15.3‡	
			12.2	10.3		16.2	
			11.6	10.4		16.7	
				10.5		21.0§	
				8.8		24.0	
				9.0			

---

* Dry milk with water to equal fresh milk.

† Fresh milk concentrated to half its volume at low temperature.

‡ One-half the amount of water.

§ No water.

reported by Sherman and his coworkers (27). On a bread and milk ration in which milk furnished one-fifth of the total calories young rats made normal growth but the females usually failed to breed and no young were raised. When ground whole wheat replaced white bread or patent flour young were successfully suckled at the cost of considerable loss in weight of the mother and grew at less than normal rate. When the amount of milk was increased so as to supply two-fifths of the total calories and the rest by ground whole wheat, young were suckled without undue loss of weight on the part of the mother and showed a fully normal rate of growth. A preliminary inference from these results (which the authors did not make) that a ration in which milk supplied all the calories would show equally good or even better results is not justified by the behavior of our animals, and it is evident that ground whole wheat supplied something which milk does not contain in adequate amounts if at all. In contrast with these results is the experiment of McCollum (28), in which a pig taken at weaning was kept on a ration of cow's milk for a period of 17 months, during which time the animal grew and seemed to be properly nourished. At 13 months she pro-

TABLE II.

Food Intake of Rats on Dry Milk and Dry Milk Foods.

The figures represent the average daily ingestion of two rats over 10 day periods beginning at the time of weaning. The animals were mated unless otherwise indicated. Rat 3018 was alone.

No. 3018♂.	Nos. 3019♂, 3020♂.	Nos. 3069, 3073.	Nos. 3070, 3074.	Nos. 3056, 3057.	Nos. 3064♂, 3066♂.	Nos. 3067, 3071.	Nos. 3068, 3072.
3.5(?)	6.5(?)	7.8	6.1	7.7	9.8	8.7	7.7
4.4	10.2	11.9	8.8	7.9	13.5	13.6	11.1
5.9	11.6	12.8	9.0	8.6	17.4	16.4	13.4
6.1	15.3	14.0	10.2	10.4	19.3	15.4	14.5
6.5	13.8	14.0	9.7	7.7	15.7	15.0	15.0
6.9	14.6			6.5	16.8	18.9	15.4
7.6	18.2			6.2	16.4	18.2	15.3
6.4	20.2			3.0	16.5	19.2	16.7
7.5	11.6				17.7	18.7	19.0
9.7	14.9				18.0		19.4
					11.2		

duced eight living and two dead pigs and successfully brought the young to an average weight of 17 pounds. Some additional iron was furnished by the drinking water and by the rusted parts of milk cans.

In a search for the cause of the inadequacy of the fresh milk ration various additions were made. Since milk is not so rich in water-soluble vitamine (29) as the original experiments of Hopkins (11) seem to indicate, and inasmuch as an inadequate amount of this substance has been shown to lead to infertility (30), a small amount (0.5 cc.) of yeast filtrate (after autolysis) was daily added to the milk fed to each of two pairs of animals (Nos. 3025, 3016, 3015, 3026). Yeast proteins were thus supplied in addition to vitamine. This supplement slightly increased the rate of growth in the males temporarily, and one of the females (No. 3026) became pregnant. The size of the litter could not be determined since only one (dead) and part of another were found. Subsequent addition of 0.5 gm. of yeast to the daily ration of this pair was without permanent effect. The abundant supply of water-soluble vitamine in wheat embryo suggested its use as a supplement to milk and for these preliminary experiments the wheat embryo was merely ground and ether-extracted since the fat is toxic for rats (10, 31), and in this powdered form 1 gm. per day was stirred into the milk supplied to Rats 3048 and 3049. Aside from a slight increase in food consumption and a temporary improvement in the rate of growth of the female this addition has thus far not yielded any positive results. Among many other procedures to be tried in connection with the problem of water-soluble vitamine is the addition to milk of further amounts of the same vitamine that it contains, and of preparations of the vitamine from other sources containing as little as possible of other ingredients.

Since milk has a low iron content, the addition of proportionate amounts of a solution of iron citrate[2] was made in several cases, in Rats 3062 and 3063 from the very beginning. The male made an excellent growth record for 120 days, losing thereafter; the female became pregnant and cast a litter of six which were all dead on the following day. Later she cast another litter of four which had disappeared by the next day. The growth of this pair was by far the best of any on raw milk and their milk consumption was generally somewhat above that of any others on raw milk. In certain of the animals as indicated in the charts additions of iron and of iron in conjunction with yeast were without effect. The addition of sodium either as chloride or citrate[3] and the supplying of casein fed separately were alike ineffective. The animals receiving a salt addition showed an especially low food intake and correspondingly poor growth.

---

[2] 0.5 cc. of a 10 per cent solution per 100 cc. of milk.

To throw further light on whether the concentration of nutrients in milk was too small to supply adequate food in the volume that a rat could consume, two animals, Nos. 3085 and 3086, were fed on fresh milk concentrated to half its volume by evaporation under diminished pressure below 50°C. The male made an exceptional growth but died after a very small loss in weight from causes which autopsy did not reveal. The growth of the female was not very different from that of those on ordinary milk, but although a male that had been on stock food was later mated with her no pregnancy resulted within 26 days. The food intake of these animals in milk solids was comparable to the intake of dry milk in other animals.

The results obtained with dry milk are shown in Chart 3, and some of the food consumption records in Table II. Both males and females show a much more satisfactory growth than on fresh milk, the males being practically normal, the females somewhat below normal after about 75 days. None of the curves shows so pronounced a slowing of growth at 50 to 100 days as do those of the animals on fresh milk. The short period of decline in weight of Rats 3018 and 3019 with subsequent partial recovery accompanied a decrease in food consumption which in the case of No. 3019 at least can be ascribed to intestinal infection, with diarrhea. This condition was not observed with No. 3018, and No. 3036, which was mated with No. 3018 at that time, showed no decline in weight. These growth results are in partial agreement with earlier experience. Lane-Claypon (32) found that rats showed a slightly more rapid gain in weight on dry milk than upon boiled milk and these in turn a rather more rapid gain than those fed on raw milk. However, bread was a part of the ration. Winfield (33) fed rats on dry milk exclusively and found that growth in both sexes fell below normal when one-half to two-thirds of the adult weight was reached and was only renewed when a mixed dietary was put in place of dry milk. There are no data on reproduction. The considerably better growth performance of rats on dried milk as compared with fresh milk is primarily a result of their getting more food. The limitation which liquid milk placed on food consumption is shown in the food record of Rats 3027 and 3028 (Table I) which were fed dry milk moistened with seven times its weight of water so as to approximate the composition of fresh milk. While their food intake in total solids was higher than that of animals on fresh milk it increased when the amount of water was halved and almost doubled when the water was omitted entirely. There was no reproduction among these rats on dry milk even though they were mated variously.

---

[3] 1 cc. of a 12.5 per cent solution per 100 cc. of milk.

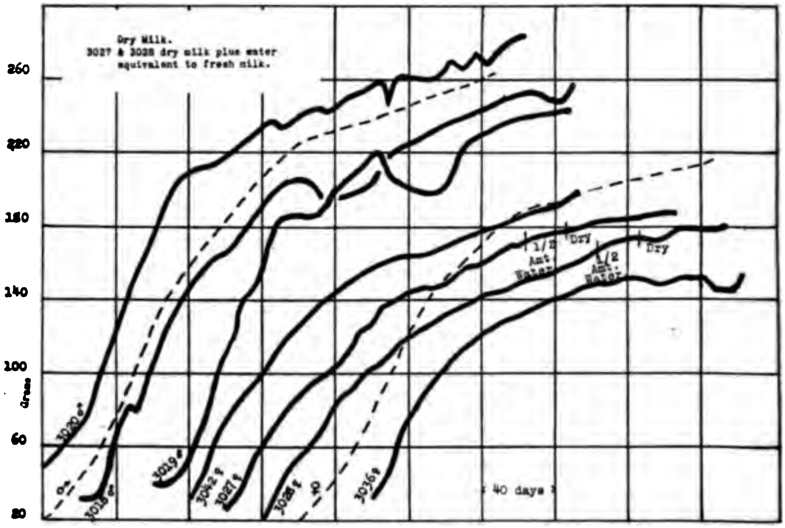


CHART 3.

The retardation of growth between 50 and 100 days in females on dry milk, and a similar behavior of both males and females on fresh milk, indicate the possibility of a requirement for new as well as for additional food. Rats arrive at sexual maturity at about the age of 60 days; the thymus undergoes atrophy and the gonads especially in the female develop rapidly during the next 50 days. If we employ the terminology of growth cycles as developed by Robertson, this age in the rat falls in the early portion of the third growth cycle, the beginning of which he has found for man (34) and for mice (35) to precede the age of puberty. The transition from one cycle to another he considers a critical period and in his own and other investigations various substances have been shown to influence certain types of growth differently. The results of milk feeding in rats, especially in the female, suggest a qualitative as well as a quantitative inadequacy of milk to meet the demands of the third growth cycle. It is not impossible that milk might contain substances inhibitory to growth in the third cycle.

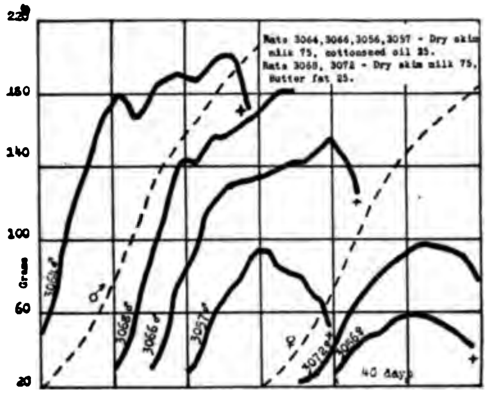


CHART 4.

Among the modifications of the dry milk ration which it was thought might throw some light on the question were those involving fat and fat-soluble vitamine. If the latter had been partially destroyed, sufficiently perhaps to affect reproduction, then the use of skim milk powder to which an equivalent amount of butter fat was added should be more satisfactory. The animals on this ration, Nos. 3068 and 3072, Chart 4, did no better than others on dry whole milk, the female especially making very poor growth. Several animals were also placed on a ration of dry skim milk 75 per cent and cotton-seed oil 25 per cent. Three males, Nos. 3057, 3064, and 3066, Chart 4, made satisfactory growth for 40 to 50 days, at which point all the curves "break" and while two of them continued to grow slowly they all died in 80 to 125 days. The record of the female, No. 3056, Chart 4, on this ration is very poor. The content of this ration in butter fat is about 1 per cent and from the figures of food intake of the two groups, Nos. 3064, 3066 and 3056, 3057, it is seen that none of the four animals ever obtained 0.1 gm. of butter fat per day, an amount on which Osborne and Mendel have recently reported (36) good growth and maintenance for about 240 days. It is probable that these four animals succumbed because of lack of fat-soluble vitamine although there were never signs of xerophthalmia (37) and aside from emaciation only roughened coats. On skim milk powder alone rats succumb quickly following little or no growth (Rats 3043, 3050, 3051, Chart 5), an effect which, in the light of our results, could be ascribed in part at least to a deprivation of fat aside from a lack of fat-soluble vitamine.

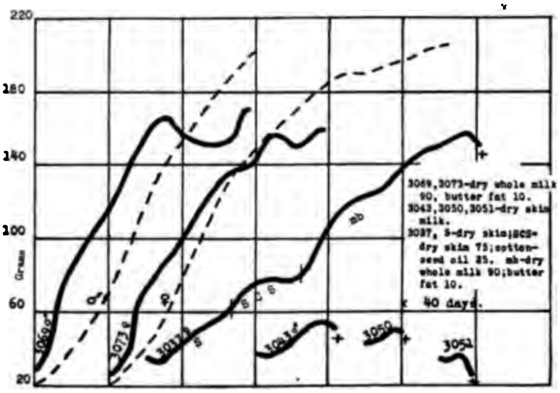


Chart 5.

The effect of an increase in the amount of fat and fat-soluble vitamine was also determined by feeding a ration containing dry milk 90 per cent and butter fat 10 per cent. The animals on this ration, Nos. 3069 and 3073, Chart 5, made good growth for about 80 days and the female cast a litter the size of which could not be recorded, since only one (dead) was found; at autopsy the uterus showed eight placental scars but the mother's weight had not been affected so far as the weekly weighings showed. Both curves broke after 70 to 90 days of this ration. Another animal, No. 3037, Chart 5, which had been transferred from skim milk powder to the same plus 25 per cent of cotton-seed oil with continued very slow growth, was placed on whole milk powder plus 10 per cent butter fat. Growth was resumed at a normal rate on the lower level but this animal died after about 100 days on this ration.

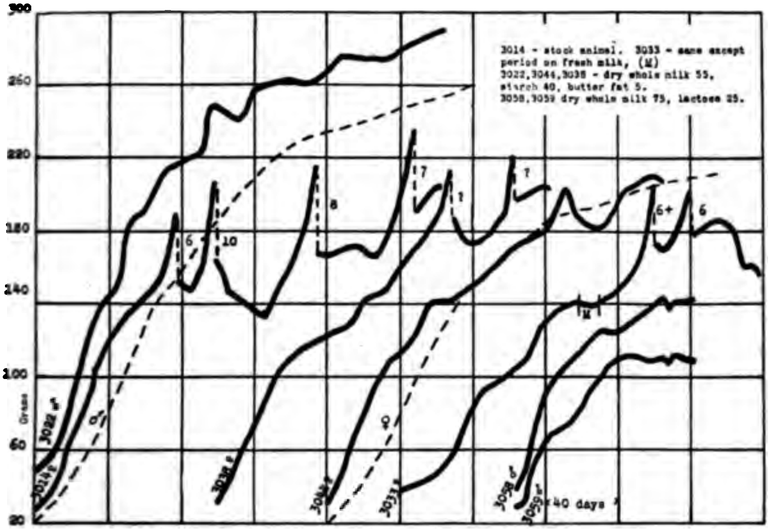


CHART 6.

A further modification of the dry whole milk ration gave interesting results. This consisted of dry milk 55, starch 40, and butter fat 5 per cent, and upon it three rats (Nos. 3022, 3038, and 3044, Chart 6) made entirely satisfactory growth over the whole period of the experiment. The curves of the females do not show the marked break at 50 to 100 days which many on milk alone exhibit. One of the females cast two litters but only one of the young animals was found. The weight curve of the other suggests a litter but none was found. As this ration contains only 55 per cent of the protein, salts, and water-soluble vitamine of dry milk and about 70 per cent of its fat content, it is somewhat analagous to the milk food[4] used by Osborne and Mendel (17) on which they reported entirely normal growth and reproduction. However, in their charts[5] it is seen that in the two animals discussed reproduction did not occur until relatively late, in one at about 400 days of age, close to menopause, in the other at 185 days of age, while breeding should normally begin at 90 to 100 days. It is not stated whether these animals were mated throughout the period of observation. Without exception the females of our small colony on stock food have had their first litters when under 125 days of age and the curve of No. 3014, Chart 6, shows typical behavior in this respect. A food can hardly be considered entirely successful when reproduction is delayed beyond the normal age at which it should begin, but it is extremely interesting that animals are able to reproduce on a 60 per cent dry milk ration with lard and starch when they do not do so on dry milk alone. In contrast with our less successful animals on 55 per cent milk powder with starch and butter fat are two others, Nos. 3058 and 3059, Chart 6, which were placed on a ration of 75 per cent dry milk and 25 per cent lactose. In this ration the proteins, salts, fats, and vitamines are more abundant than in the other, the principal difference being a substitution of lactose for starch. But these rats (males) stopped growing, one at 112 gm. the other at 143 gm., and this despite the fact that lactose occupies a unique position among carbohydrates, while starch is not believed to carry any vitamines (38) or to possess any peculiar virtues. Our data are as yet too limited even to justify hypothesis but the results of Osborne and Mendel just quoted as well as those of Sherman mentioned above point in the same direction. A tentative explanation would be that milk possesses an inhibitory agent for adolescent growth and reproduction, the effect of which is partially obviated by dilution with other foodstuffs and that these foodstuffs, in particular starch (and lard was also used by Osborne and Mendel), provide a requisite which milk does not possess. What these substances might be is a matter of conjecture, but starch contains phosphorus in organic combination with a carbohydrate the nature of which is unknown (39, 40).

[4] Whole milk powder 60, starch 12, lard 28 per cent.

[5] Osborne and Mendel (17), p. 320.

The possibility of transient or permanent infection of the lungs, alimentary tract, or other organs, with a resultant subnormal efficiency, is a very real one especially when the food is inadequate. The susceptibility of rats to lung disease militates somewhat against their value as a laboratory animal, but in our experience the presence of this infection is not always evidenced by the animal's capacity for growth and reproduction. Thus Rat 3014 whose curve is shown in Chart 6 had very bad lungs, which probably accounts for her low weight, though her reproduction record is fairly satisfactory. Similar discrepancies are shown in Table III. According to McCollum[6] rats on a milk diet have foul cages, a fact not entirely borne out by our experience, perhaps because our animals were on an exclusively milk diet from weaning. The cages of animals on fresh milk required more frequent renewal than others but except in a few cases of diarrhea the feces were well formed and without abnormal odor.

Several attempts have been made to determine whether the incapacity for reproduction on milk rations resides in the male or the female, but the introduction for limited periods of males or females from stock food into cages with animals on milk has not given any positive results. Rat 3033, Chart 6, may be cited. From stock food without previous mating she was transferred at the age of 125 days to a cage with a male on raw milk. After 11 days she was returned to a stock cage and mated. 30 days thereafter she cast a litter of six which she did not raise. Impregnation had evidently not been accomplished by the milk-fed male but after her return to the stock cage. A second litter followed immediately (after 3 weeks) which she raised successfully, but the choice of this animal for a test was perhaps unfortunate because of some pathological condition evidenced by her subsequent decline in weight. Her ovaries were found to be below normal in weight (Table III).

Many of the animals whose curves are given have not been continued on experiment since it was desired to examine them post mortem. In Table III are given the weights of the sex glands together with brief autopsy findings. The weights of the thymus would also have been very desirable but it was not possible to

---

[6] McCollum (28), p. 76.

TABLE III.

No. of animal.	Age.	Final weight.	Weight of gonads.	Normal weight of gonads.*	Difference.	Ration.	Autopsy.	
							State of lungs.†	Remarks.
	days	*gm.*	*gm.*	*gm.*	*per cent*			
Males.								
3001	367	222	2.17	2.30	− 5.7	Stock.		Small intestine somewhat inflamed.
3017	271	212	2.56	2.25	+ 6.8	"	++	
3046	206	250	2.45	2.45	± 0.0	"	+	
3015	262	177	2.45	2.02	+21.3	Raw milk.	+++	Abundant fat.
3016	269	229	2.16	2.33	− 7.3	" "	+++	
3019	255	239	2.80	2.40	+16.7	Dry "	+	Fat. Fine condition.
3022	257	292	2.29	2.64	−13.3	55 per cent dry milk.	+	Fat very abundant.
3045	201	136	1.82	1.70	+ 7.1	Raw milk.	++	
3058	169	159	2.04	1.88	+ 8.5	Dry " + lactose.	+	
3059	169	85	1.03	1.08	− 4.6	" " + "	++	Emaciated.
3063	164	205	2.14	2.21	− 3.2	Raw " + Fe.		
3064	144	171	1.05	1.99	−47.2	Skim " powder + cotton-seed oil.	+++	
3066	140	125	1.65	1.58	+ 4.4	" " " + " "	++	
3068	129	187	1.98	2.10	− 5.7	" " " + butter fat.		
3069	145	170	2.26	1.98	+14.1	Whole " " + " "		
Females.								
3006	374	200	0.071‡	0.049	+44.9	Stock.	++	
3014	276	192	0.065‡	0.049	+32.6	"	+++	
3029	268	205	0.051‡	0.049	+ 4.1	"		Fat abundant.
3033	223	155	0.023‡	0.047	−51.1	"	+	

---

* Donaldson (41), p. 101.

3025	245	175	0.012	0.048	−75.0	Raw milk.	+	Liver slightly hemorrhagic.
3026	238	157	0.036‡	0.047	−23.4	" "		Abundant fat.
3035	204	106	0.015	0.032	−53.1	" "	++	
3038	216	193	0.038‡	0.049	−22.4	55 per cent dry milk.	+	Liver hemorrhagic; uterus inflamed.
3041	201	93	0.015	0.022	−31.8	Raw milk.		
3044	213	201	0.026‡	0.049	−46.9	55 per cent dry milk.	+	Abundant fat; intestine and uterus inflamed.
3060	155	83	0.014	0.016	−12.5	Raw milk + NaCl.		
3061	155	94	0.012	0.024	−50.0	" " + NaCl.		
3062	164	134	0.037‡	0.045	−17.8	" " + Fe.	+	Uterus inflamed.
3072	126	74	0.016	0.013	+23.1	Skim " powder + butter fat.	++	
3073	142	160	0.047	0.048	− 2.1	Whole " " + " "		
3074	154	140	0.026	0.046	−43.5	Raw "		
3086	135	153	0.030	0.047	−36.2	Concentrated milk.	+	

obtain them at this time. The sex glands were dissected and weighed as indicated by Donaldson (41), but without using a moist chamber, and the secretion of the male gonads was usually also examined under the microscope. With one exception the weights of the testes were within normal limits; sperm were always present and if the examination was made immediately after the death of the animal they were seen to be motile. The ovaries, on the other hand, showed marked variation and with one exception the ovary weights of the animals on milk rations were below normal, even 50 per cent and more. The conclusion seems justified, therefore, that an exclusively milk ration fails to supply something which is specifically required for reproduction in the female, being at the same time adequate, in so far as sperm production goes, for the male. Further breeding trials must be made to determine the complete efficiency of the male.

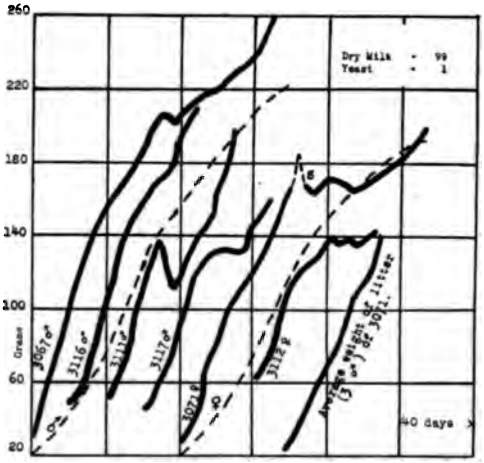


Chart 7.

One further series of animals should be mentioned. The observations in experiments being conducted on mice that an addition of yeast to a dry milk ration effected better reproduction than had been secured, as well as the partially successful result of yeast filtrate addition to fresh milk in rats (Chart 2), suggested the addition of dry yeast to whole milk powder. Rats 3067 and 3071, Chart 7, to which this was fed (1 per cent yeast) are the only animals of the milk-fed series that have thus far not only made satisfactory growth themselves but also have successfully raised young. Of the original litter of eight, five (died (?) and) were eaten by the parents, but the three remaining have grown fairly well on the same ration as the composite curve indicates. There has not been another litter although the parents are still mated. The growth of other rats on this ration is shown in Chart 7 and that of rats on whole milk powder containing 5 per cent yeast in Chart 8. The performance of these animals may show whether yeast really supplies something unique. The results are at least interesting in comparison with those of Osborne and Mendel (42) on four males fed yeast as their sole source of protein and water-soluble vitamine, which grew normally but remained infertile.

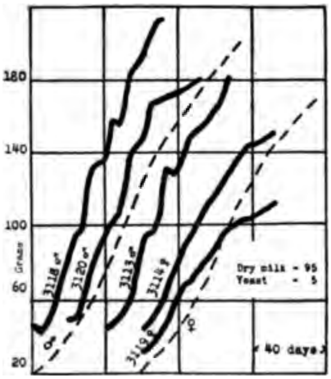


Chart 8.

A word should be said with reference to the factor of variability. Since all these experimental animals were taken from weaning, the usual period during which they are allowed to grow to a weight of 50 or 60 gm. before being placed on an experimental ration is omitted. In this period it is possible to detect subnormality more readily than in the earlier stage before weaning. While we used no animals that were subnormal it is quite possible for the above reason that a larger number of animals will be required to establish these results than would otherwise be necessary. It is safe to say that variability is in general too little considered in this type of investigation.

SUMMARY.

In these preliminary experiments young rats taken at weaning were placed on various rations consisting primarily of cow's milk, fresh and desiccated. On fresh milk rats made good initial growth but beginning between the 50th and 100th day of life, especially in the females, there was a decided retardation. There was no reproduction. The addition of yeast filtrate temporarily increased the rate of growth and one female became pregnant, but the litter was eaten. Addition of wheat embryo also increased the rate of growth with no further effect thus far. When milk was supplemented with iron citrate growth was much more satisfactory but reproduction was not successful. The growth failure was in part the result of the dilute form of the food, for on dry milk rats made much better growth and their average food consumption in milk solids was considerably greater than that of those fed on fresh milk. The females, however, remained somewhat below normal after 75 days and again there was no reproduction. Neither substitution of dry skim milk to which butter fat was added equivalent to that in dry whole milk nor the addition of 10 per cent of butter fat to dry whole milk was successful.

On a ration containing 55 per cent of dry whole milk, 40 per cent of starch, and 5 per cent of butter fat, both male and female rats made practically normal growth, and the females bore young but did not raise them. A similar ration has been used by other investigators who secured not only satisfactory growth but also reproduction, while other investigators have, like ourselves, not had success with whole dry milk alone. Dilution of dry milk by 25 per cent lactose resulted in poor growth and no reproduction. The gonads of many of the animals were weighed; the testes were with one exception of normal weight and contained motile sperm; the ovaries on the other hand were much under normal weight, even 50 per cent and more.

Possibly milk is lacking both quantitatively and qualitatively in substances necessary for successful adolescent growth and reproduction, especially in the female, and it may contain substances that are inhibitory to the growth of an animal in the third or mature growth cycle. A ration containing dry milk and 1 per cent of yeast is the only one on which normal growth and partially successful reproduction were obtained, and the growth of rats on this ration and on one containing 5 per cent of yeast is thus far practically normal. Whether yeast supplies something unique remains to be seen. The factor of variability necessitates data on a larger number of animals before definite conclusions can be drawn.

BIBLIOGRAPHY.

1. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.
2. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 1.
3. Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxvii, 223.
4. McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 287.
5. Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1919, xxxviii, 147.
6. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.
7. Drummond, J. C., *Biochem. J.*, 1916, x, 89.
8. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167.
9. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423.
10. Drummond, J. C., *Biochem. J.*, 1919, xiii, 95.
11. Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.
12. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.
13. Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1919, xxxviii, 257.
14. Mitchell, H. H., *J. Biol. Chem.*, 1919, xl, 399.
15. Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1919, xl, 449.
16. Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.
17. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.
18. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.
19. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379; 1916, xxiv, 37.
20. Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.
21. Drummond, J. C., *Biochem. J.*, 1919, xiii, 81.
22. Barnes, R. E., and Hume, E. M., *Biochem. J.*, 1919, xiii, 306.
23. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33.
24. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxix, 29.
25. Lane-Claypon, J. E., Milk and its hygienic relations, London, New York, Bombay, and Calcutta, 1916.
26. Hatai, S., *Am. J. Anat.*, 1917, xxi, 23.
27. Sherman, H. C., Rouse, M. E., Allen, B., and Woods, E., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 9.
28. McCollum, E. V., The newer knowledge of nutrition, New York, 1919, 70.

29. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537; 1920, xli, 515.
30. Drummond, J. C., *Biochem. J.*, 1918, xii, 25.
31. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105.
32. Lane-Claypon, J. E., *J. Hyg.*, 1909, ix, 233.
33. Winfield, G., *Gt. Brit. Local Gov. Bd. Food Rep.*, 1918, xxiv, 139.
34. Robertson, T. B., *Am. J. Physiol.*, 1915, xxxvii, 1.
35. Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363.
36. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.
37. Bulley, E. C., *Biochem. J.*, 1919, xiii, 103.
38. Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.
39. Thomas, A. W., *Biochem. Bull.*, 1914, iii, 403; 1915, iv, 379.
40. Northrop, J. H., and Nelson, J. M., *J. Am. Chem. Soc.*, 1916, xxxviii, 472.
41. Donaldson, H. H., The rat, Philadelphia, 1915.
42. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxviii, 223.

THE EFFECT OF COOKING ON THE WATER-SOLUBLE VITAMINE IN CARROTS AND NAVY BEANS.

By ELIZABETH W. MILLER.*

(From the Hull Laboratories of Physiological Chemistry and Pharmacology, University of Chicago, Chicago.)

(Received for publication, July 14, 1920.)

The increasing use of highly refined food products, in which the content of vitamine is low, makes correspondingly great the necessity of including, in the diet, foods which are especially rich in these indispensable factors. There is considerable evidence, derived from animal feeding experiments, to show that some of the common vegetables are valuable as sources of the water-soluble vitamine. Among those that have been found to contain relatively large amounts of this substance are cabbage,[1, 2] spinach,[2] potato,[3] navy bean,[4] carrot,[5] and onion, turnip, beet, and tomato.[6] But in most of these experiments the uncooked vegetables, fresh or dried, have been used. It has been found that in cooking there is a considerable loss of protein and other constituents by solution in the cooking water. The vitamine content may also be diminished to such an extent that the cooked and uncooked vegetables cannot be compared as sources of this substance. It is therefore desirable to obtain information concerning the effect of the various processes of preparation on this important constituent. In the experiments reported here the

---

[*] Richards Fellow in Household Administration.

[1] McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187.

[3] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxvi, 197.

[4] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

[5] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 171. Denton, M. C., and Kohman, E., *J. Biol. Chem.*, 1918, xxxvi, 249.

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxix, 29.

vitamine was determined in carrots and navy beans cooked by methods commonly used in the household.

Quantitative determinations of the water-soluble vitamine in foods have been made either by means of growth experiments on animals or experiments for testing the antineuritic properties, as measured by the preventive or curative effect. Most investigators consider that the water-soluble, growth-promoting vitamine and the antineuritic substance are identical. Results obtained from both methods of experimentation have been reported to show the effect of high temperatures on the water-soluble vitamine in food. These results, however, show considerable variance.

Most of the evidence agrees that the vitamine is not destroyed by heating for considerable periods of time at temperatures of 100°C. or lower. McCollum and Davis[7] found that wheat embryo, milk whey, and crude lactose from which the vitamine was not entirely removed could be heated for 1 hour at 15 pounds pressure (120°C.) without apparent loss of the growth-promoting property. McCollum, Simmonds, and Pitz[4] detected little, if any, diminution when navy beans were heated in a moist condition at 120°C. for 1¼ hours.

Daniels and McClurg[8] conclude from results obtained in feeding young rats that cooking cabbage and beans (navy and soy) did not destroy the water-soluble vitamine to any great degree. They cooked the beans in three ways—in water at 100°C., in dilute alkali at 100°, and at 120° for 15 to 20 minutes. However, the diets fed contained for every 100 gm. of dry food the equivalents of 50 gm. of navy beans, or 55 gm. of soy beans, or 250 gm. of fresh cabbage which undoubtedly supplied an excess of the growth-promoting vitamine. McCollum, Simmonds, and Pitz[4] found that 25 gm. of navy beans for 100 gm. of dry food was sufficient to supply this dietary essential. The vitamine content might have been reduced to 50 per cent of the original value and the loss not detected in these experiments.

Chick and Hume,[9] on the other hand, report that heating wheat germ at a mean temperature of 113°C. for 40 minutes

[7] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

[8] Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, 1919, xxxvii, 201.

[9] Chick, H., and Hume, E. M., *Proc. Roy. Soc. London, Series B*, 1917, xc, 60.

reduced its curative effect, when fed to polyneuritic pigeons, to one-half the original amount. Heating the wheat germ at 118–124°C. for 2 hours caused such rapid destruction that its antineuritic activity was less than one-fourth that of the untreated material. There was no significant decrease by heating at 100°C. for 2 hours.

Davis[10] finds that the ordinary pigeon ration of corn, Kaffir corn, barley, and split peas when heated for 2½ hours at 120°C. no longer protects against polyneuritis. In fact the mixture treated thus is used as the beri-beri-producing ration.

There is a similar lack of agreement in the evidence concerning the stability of the water-soluble vitamine to alkali. McCollum and Simmonds[11] report almost complete destruction when an extract of navy beans was made alkaline to 0.28 per cent with sodium hydroxide and heated under a reflux for 1 hour. Daniels and McClurg,[8] on the contrary, found no apparent loss when an alkaline water extract of beans was heated under the same conditions. However, the material used by McCollum was extracted from the beans with alcohol, after a preliminary extraction with ether. There is evidence which indicates that the vitamine exists naturally in some protective combination which is broken up by the treatment with ether and extraction with alcohol.[12]

Osborne, Wakeman, and Ferry[13] digested brewery yeast with ten times its weight of 0.1 N sodium hydroxide solution for 21½ hours and then heated it on the water bath for 2 hours. After this treatment it still produced as prompt recovery when fed to rats that had begun to lose weight on a diet practically vitamine-free as an equivalent amount of untreated yeast. They conclude that the water-soluble vitamine is more stable to alkali than has usually been supposed. But in this case also the yeast was not subjected to extraction with ether before heating with the alkali.

---

[10] Davis, M., *J. Home Econ.*, 1920, xii, 206.

[11] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

[12] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

[13] Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

A much simpler and more delicate method for the quantitative determination of the water-soluble vitamine has been recently suggested by Williams.[12] He found that yeast cells require for their growth a substance which he concludes from evidence he has obtained is identical with the substance which in animal nutrition prevents polyneuritis. He showed that the rate of reproduction of a single yeast cell paralleled, within limits, the concentration of the vitamine which was added to the medium in which the yeast was grown. Bachmann[14] also found that certain yeasts would not ferment unless some substance rich in water-soluble vitamine was added to a medium containing inorganic salts and sugar. She also suggested that such yeast might be used to determine quantitatively the amounts of vitamine in various substances.

Since there is such apparent disagreement in the results reported by different investigators as to the effect on the vitamine content of subjecting foods to ordinary cooking processes, it seemed advisable to carry out experiments using the yeast method. Two vegetables, carrots and navy beans, were cooked by the methods most commonly used and the vitamine in the cooked vegetable and in the cooking water was determined separately. Carrots were cooked at 100°C. for 30 minutes and canned at 10 pounds pressure for 45 minutes. For comparison the vitamine content of the raw carrots was also found. Since dried legumes require a longer cooking period than other vegetables, experiments were carried out with navy beans. The beans were cooked by three methods: in distilled water at 100°C. for 1½ hours, in 0.5 per cent sodium bicarbonate solution for 1 hour and 10 minutes, and in distilled water at 120° for 30 minutes.

EXPERIMENTAL.

Method of Determining the Vitamine.—A quantitative method of determining the amount of vitamine in a solution was perfected by Williams[12] and Swoboda.[15] The average growth of single yeast cells, when different volumes of a vitamine solution were added to the medium in which the yeast was grown, was found to have a definite quantitative relation to the amount of vitamine added. The best comparative results were obtained when the growth was counted at the end of 18 hours. When no vitamine was added to the medium an average growth of four to eight cells was obtained from a single cell. Since this method has been given in detail by Williams[12] and Swoboda[15] it will be described only briefly here.

---

[14] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

[15] Swoboda, F. K., unpublished experiment.

The medium used for the growth of the yeast contained the following substances dissolved in 1 liter of redistilled water.

20	gm.	of sucrose (Kahlbaum).
3	"	" $(NH_4)_2SO_4$ (Merck).
2	"	" KH_2PO_4 (Merck).
1.5	"	" asparagine (Kahlbaum).
0.25	"	" $CaCl_2$ (Central Scientific).
0.25	"	" $MgSO_4$ (Baker).

25 cc. portions of this solution were measured into flasks and autoclaved at 10 pounds pressure. After cooling, 3 and 5 cc. quantities of the solution to be tested were added to the medium and when necessary redistilled water to make up the volume to 30 cc. Duplicate solutions were prepared in each case and blanks run for controls. Finally 1 cc. of a suspension of Fleischmann's yeast in redistilled water was added to each solution. This suspension was made by shaking a piece of yeast about the size of a small grain of wheat in 30 cc. of water. Hanging drops were then made from each solution with a sterile pen.

The slides were examined immediately to count the single cells. Only those drops which contained three or less than three single cells were considered. The slides were then put into an incubator kept at 30°C. A second reading was made at the end of 6 hours to verify the first record and to detect any cells which did not grow. Finally a count was made of the cells in each drop at the end of 18 hours.

Preparation of Vitamine Solution from the Vegetables.—In using the yeast method the vitamine must be in solution. It was necessary therefore to extract the vitamine from the vegetable quantitatively. Dixon and Atkins[16] found that the plant juices could be removed from the tissue most effectively by freezing the material and then pressing. This method was adopted for obtaining the juice from raw carrots. To make certain that freezing has no effect on the vitamine a yeast extract of known activity was frozen and kept at −7°C. for 2 weeks. The results given in Table I show that there was no loss in activity caused by this treatment.

---

[16] Dixon, H. H., and Atkins, W. R. G., *Sc. Proc. Roy. Dublin Soc.*, 1912–13, xiii, 422. Gortner, R. A., Lawrence, J. V., and Harris, J. A., *Biochem. Bull.*, 1916, v, 139.

TABLE I.

Effect of Freezing on the Vitamine.

	Quantity added.	No. of cells growing.	Maximum.	Minimum	Average.	Average of duplicate determinations.
	cc.					
Before freezing...........	3	20	170	40	126	
" "	3	18	192	30	142	134
After "	3	14	160	80	128	
" "	3	20	200	80	132	130

TABLE II.

Lack of Adsorption of Vitamine by Berkefeld Filter.

	Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.
	cc.					
Dilute solution.						
Unfiltered...........	3	17	71	22	37.7	
	3	21	60	23	33	35.3
Filtered.............	3	25	56	25	35	
	3	16	60	16	36	35.5
Concentrated solution.						
Unfiltered...........	5	16	352	130	218	
	5	23	400	150	226	222
Filtered.............	5	18	400	112	215	
	5	14	400	120	212	213

It is also necessary that the vitamine solution should be sterile since other organisms introduced into the medium interfere with the growth of the yeast. To avoid sterilization by heat the vitamine solutions prepared from the vegetables were run through a Berkefeld filter. Tests were made with yeast extracts of different concentrations which had previously been sterilized by autoclaving to find if any of the vitamine was adsorbed by the filter. The results given in Table II show that there was no loss of the active substance by this treatment.

Preparation of Carrots.—Carrots were purchased from the market in the fall. They were mature but fresh and still retained some of the green stems. In scraping off the skin, care was taken to remove as little of the vegetable as possible. To obtain uniform samples for the separate experiments the carrots were cut lengthwise into thirds. One third of each carrot was then used as described below for the determination of vitamine in raw carrots, another third was cooked at 100°C., and the other portion was canned at 10 pounds pressure.

Extraction of Juice from Uncooked Carrots.—The carrots were ground in a meat chopper, packed in a metal cylinder, and frozen at −7°C. The solid block of vegetable was then shaved to a fine snow in a Kossel[17] hasher. After thorough mixing, two portions of this finely divided material were immediately weighed out for determination of moisture. A third portion to be used for the determination of vitamine was put into a tincture press and the juice expressed. In order to remove the vitamine from the vegetable quantitatively, the dry residue was covered with water, allowed to stand 15 minutes, and again pressed. This was repeated six times when the extraction proved to be complete. The fifth extract when tested gave an average growth of only three cells per gram of the original fresh carrot. A sixth extraction was made to assure complete removal of the vitamine.

The combined filtrate and washings from 45.5 gm. of carrots were diluted to 2 liters, filtered through a Berkefeld filter, and used for the determination of vitamine. It was necessary to dilute the solution sufficiently to keep the number of yeast cells produced in a single colony within a range that could be counted. When the growth was heavy a micrometer disk was used to aid in counting. The colonies sometimes grew in three dimensions. It was necessary then to make an estimation of the number of cells.

---

[17] Kossel, A., *Z. physiol. Chem.*, 1901, xxxiii, 5.

Carrots Cooked at 100°C.—A second portion of the fresh carrots was cut into ½ inch cubes, and 200 gm. were weighed out and cooked in distilled water for 30 minutes. The cooking water was drained off and diluted to 1 liter. The cooked carrots were mashed in a mortar and the juice was obtained by pressing as in the case of the frozen carrots. The juice and washings from the cooked carrots were diluted to 6 liters.

Canned Carrots.—326 gm. of the same lot of carrots were diced and packed in a pint jar. Boiling water was added to fill the jar and the vegetable was then subjected to 10 pounds pressure for 45 minutes. The water in the can was drained off and diluted to 3 liters. The entire 326 gm. were too bulky to handle conveniently in the press, so, after mashing the vegetable and mixing, 79 gm. (the equivalent of 74 gm. of raw carrots) were weighed out. The juice from this amount was expressed and diluted to 3 liters.

Navy Beans Cooked at 100°C.—30 gm. of navy beans were cooked in 150 cc. of distilled water for 1½ hours. The cooking water was diluted to 1,200 cc. It was found more difficult to remove the vitamine quantitatively from the beans than from the carrots. For each extraction the bean pulp was covered with water and warmed gently for at least 1 hour before pressing. Repeating this seven times finally extracted all the vitamine. The juice and washings from the 30 gm. of beans were diluted to 1,200 cc.

Navy Beans Cooked in Dilute Alkali.—30 gm. of navy beans were cooked in 120 cc. of 0.5 per cent sodium bicarbonate solution for 1 hour and 10 minutes. The excess of alkali in the cooking water was neutralized to phenolphthalein with 0.1 N phosphoric acid. It took just 3.4 cc. of acid for the entire 1,200 cc. of solution. The extract from the beans cooked in the bicarbonate solution was already acid to phenolphthalein. Most of the alkali was therefore neutralized by the natural acids of the vegetable.

Navy Beans Cooked at 120°C.—30 gm. of a new lot of beans were cooked in 150 cc. of distilled water under 15 pounds pressure for 30 minutes. These beans were very soft and considerably broken. This accounts for the fact that in this case 70 per cent of the vitamine was found in the cooking water.

For comparison 30 gm. of this same lot of beans were cooked at 100°C. for 1½ hours. The cooking water and the extract of the vegetable were in each case made up to 1,200 cc.

Discussion of the Method.

It will be seen by consulting the tables that the maximum and minimum growth from single cells in a given experiment shows considerable variation. However, the majority of the colonies were close to the average so that by inspection of the results an estimate could be made that was close to the actual average. For example, a slide in which the maximum growth was 96, the minimum 10, and the average 42, 17 out of the 26 colonies had from 35 to 50 cells.

It is not possible always to account for the unusually high or low values. Occasionally large, somewhat transparent cells were found which did not grow so vigorously as the smaller ones. When some of the cells in a drop died and became disintegrated the growth of the remaining cells was inhibited to some extent. There is also the possibility of minute particles of toxic material on the cover-slip which might check the growth. Merest traces of cleaning solution left on the cover-slip had an exceedingly toxic effect.

When proper precautions were taken, however, and when averages were made of a large number of colonies, the results showed a definite quantitative relation between the amount of vitamine added and the growth obtained. In the later work two slides were prepared from each solution making a total of 144 hanging drops for each concentration.

To illustrate how well the results check, it was found that when 3 cc. of a given vitamine solution were added to the medium the average growth from 74 colonies was 86; the average growth from 137 colonies for 5 cc. of the same solution was 136.3. Subtracting the blank which was 6.7 cells, the ratio of 79.3 to 129.6 is 3 to 4.9 instead of 3 to 5.

Two determinations were made on separate samples of raw carrots and of navy beans cooked at 100°C. The results for the carrots were 516 cells and 552 cells respectively, per gram of the fresh material, and for the beans 2,034 cells and 2,116 cells per gram. Because such concordant results can be obtained this method is of value for quantitative as well as qualitative tests. Its use requires much less time and labor than do the feeding experiments ordinarily used.

DISCUSSION.

The data given in Tables III and IV show that both carrots and navy beans are rich in water-soluble vitamine. The value obtained for dry yeast (Fleischmann's, starch-free) which is very high in water-soluble vitamine is 28,000 cells per gram. Carrots, dry weight, have a value about one-sixth as great and navy beans about one-twelfth. Osborne and Mendel[2] found that it required about five times as much dried spinach to produce satisfactory growth in young rats as it did of dried brewery yeast. Carrots, then, compare favorably with spinach as a source of water-soluble vitamine since brewery yeast contains less of this constituent than the pure Fleischmann's yeast. These results also concord with those previously reported for carrots by Sugiura and Benedict and by Denton and Kohman[5] and for navy beans by McCollum, Simmonds, and Pitz.[4] It also furnishes additional evidence as to the identity of the water-soluble vitamine and the yeast growth-stimulating substance.

Cooking carrots at 100°C. for 30 minutes caused no loss of the water-soluble vitamine. In the case of beans no comparison was made with the uncooked vegetable, but it is probable that there is no significant loss even in the longer period of cooking.

The beans cooked at the higher temperature, 120°C. for 30 minutes, showed a decided decrease in activity, a loss of 40.6 per cent. This agrees with the results of Chick and Hume[9] and of Davis.[10] There was probably a maximum loss in this experiment. It would take only a short time for the entire mass to become heated because of the small bulk. The beans were therefore subjected to the high temperature for almost the full period of heating. Then too, a very large proportion of the vitamine, 70 per cent, was in the cooking water due to the fact that the beans were overcooked and somewhat broken. The vitamine is probably less stable in solution than when it is retained in the vegetable tissue. An extract of beans heated at 115°C. for 10 minutes showed a loss of 24.4 per cent of the active substances.

TABLE III.

Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.	No. of cells for 1 gm. of original material, corrected for blank.		
						Fresh weight.	Average.	Dry weight = 11.32% of fresh weight.
A. Raw carrots. Extract from 45.5 gm. of fresh raw carrots diluted to 2 liters.								
cc.								
None.	31	17	3	7.3	7.0			
"	27	14	5	6.6				
3	26	96	10	41.8	42.9	525		
3	19	96	16	44.1				
5	26	101	20	62.2	64.8			
5	26	160	16	67.4		508	516	4,558
B. Cooked carrots. Extract from 200 gm. of cooked carrots diluted to 6 liters.								
None.	29	8	2	4.6				
"	39	7	2	3.5	4.1			
3	20	62	6	25.5				
3	13	59	12	29.5	27.5	234		
5	24	100	15	50.7	50.7	279	256	2,261
Cooking water from 200 gm. of carrots diluted to 1 liter.								
None	21	12	3	6.8	6.8			
3	26	330	126	175.5				
3	33	255	80	185.9	180.5	289		
5	23	600	125	249.5				
5	22	450	165	280.4	265.0	258	273	2,428
Total for cooked carrots							529	4,689
C. Canned carrots. Extract from 74 gm. of canned carrots diluted to 3 liters.								
None.	32	12	3	7.0				
"	26	15	2	7.8	7.4			
3	21	76	8	36.8				
3	27	50	14	32.5	34.6	367		
5	18	95	30	51.4				
5	14	104	20	56.8	54.1	378	373	3,304
Water from 326 gm. of canned carrots diluted to 3 liters.								
3	24	105	40	65.7				
3	25	150	48	71.6	68.6	188		
5	30	250	75	133.2	133.2	233	210	1,855
Total for canned carrots							583	5,159

TABLE IV.

Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.	No. of cells for 1 gm. of original material, corrected for blank.	
							Average.
A. Beans cooked at 100°C.							
cc.							
None.	32	12	3	6 0			
"	24	9	2	5.6	5.8		
Extract from 30 gm. of beans.							
3	53	116	30	59.1			
3	24	100	32	55.6	57.3	686	
5	24	200	55	100.7			
5	29	190	45	99.5	100.1	754	721
Cooking water.							
3	31	115	25	70.8			
3	42	138	40	74.1	72.4	888	
5	24	200	60	118.5			
5	41	200	50	122.5	120.5	917	902
Total							1,623
B. Beans cooked at 100°C. in 0.5 per cent $NaHCO_3$ solution.							
None.	15	10	2	6.6			
"	39	13	2	5.2	5.9		
Extract from 30 gm. of beans.							
3	38	108	13	49.6			
3	47	120	21	53.0	51.3	605	
5	27	160	32	95.0			
5	34	160	53	88.8	91.9	688	646
Cooking water.							
3	51	84	10	43.6			
3	61	90	15	49.7	46.6	542	
5	24	112	40	70.4			
5	30	120	25	78.0	74.2	546	545
Total							1,191
Loss, *per cent*							37.5

TABLE IV—*Concluded.*

Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.	No. of cells for 1 gm. of original material, corrected for blank.	
							Average.
C. Beans cooked at 100°C. Lot II.							
cc.							
None.	20	18	2	6.7	6.7		
Extract from 30 gm. of beans.							
3	23	150	28	86.1			
3	30	160	55	99.4	92.7	1,146	
5	37	320	30	133.4			
5	60	320	26	134.8	134.1	1,019	1,082
Cooking water.							
3	30	140	36	74.0			
3	52	145	18	80.6	77.3	941	
5	27	250	22	125.4			
5	35	270	36	129.3	127.3	964	952
Total							2,034
D. Beans cooked at 15 lbs. pressure for 45 min.							
None.	42	16	2	6.5			
"	18	9	2	5.0	5.7		
Extract from 30 gm. of beans.							
3	68	71	16	33.8			
3	41	67	18	32.8	33.4	368	
5	74	85	10	48.2			
5	36	96	23	47.2	47.5	334	351
Cooking water.							
3	27	118	30	69.8			
3	25	170	20	66.0	67.9	829	
5	21	240	33	117.5			
5	20	208	52	115.1	116.3	884	856
Total							1,207
Loss, *per cent*							40.6

The canned carrots heated at 115°C. for 45 minutes showed no loss of vitamine. The results indicate an apparent increase. This is doubtless due to experimental error. The entire 326 gm. was not used for extracting the juice. A small portion, 74 gm., was weighed out after the carrots had been mashed in the mortar. There was a great deal of juice and it is possible that in removing the sample an excess of the liquid was taken. The results obtained from the one slide with 5 cc. of cooking water give an average growth per gram of fresh vegetable that is 24 per cent higher than the average growth per gram obtained with the 3 cc. quantities. Since the results for the 3 cc. quantities check very closely, they are undoubtedly the more accurate. If only these averages are considered the total growth per gram of fresh material is 561 and for dry weight 4,955.

That there should be no destruction of vitamine under the conditions of this experiment is to be expected. The carrots were packed tightly in the can and there was relatively little water used, about 100 cc. It takes considerable time for heat to penetrate such a mass so that the vegetable could not have been subjected to the high temperature for long. Less of the vitamine was in solution and it may be that the greater acidity of the carrots inhibited the destructive action of autoclaving.

By cooking in 0.5 per cent sodium bicarbonate solution 37.5 per cent of the vitamine of the beans was lost. This concentration is not more alkaline than is frequently used in cooking dried legumes. While the destruction of vitamine is by no means complete it is still worth considering. It is interesting to note that both autoclaving and alkalies seem to have a destructive effect on the vitamine, just as in the treatment of proteins.

An important fact which was also noted by Daniels and McClurg[8] is that so large a proportion of the vitamine is found in the cooking water. This varies, of course, with the amount of water used. In every case except the canned carrots and autoclaved beans it is about half the total. With the beans cooked at 120°C., 70 per cent of the vitamine was extracted in the cooking water, and, with the canned carrots, 36 per cent of the total vitamine was present in the water. This emphasizes again the importance of utilizing the water in which vegetables are cooked.

SUMMARY.

1. The yeast method used for the quantitative determination of the vitamine content of vegetables has yielded good results when averages were taken of a large number of colonies.
2. Cooking carrots at 100°C. for 30 minutes caused no reduction in the vitamine. Neither was there any destruction when carrots were packed tightly in a jar and heated at 115°C. for 45 minutes.
3. Cooking navy beans at 120°C. for 30 minutes decreased the vitamine content 40.6 per cent. In this case the beans were somewhat overcooked.
4. Cooking navy beans in 0.5 per cent sodium bicarbonate solution for 1 hour and 10 minutes caused a loss of 37.5 per cent of the vitamine.
5. A large proportion of the vitamine, from 36 per cent to 70 per cent, was present in the cooking water.

In conclusion I wish to express to Professor F. C. Koch my sincere appreciation of his generosity in giving helpful suggestions and providing equipment, and to Mr. F. K. Swoboda for instruction in the use of the yeast method.

WATER-SOLUBLE B IN CABBAGE AND ONION.

By BERTHA K. WHIPPLE.

(From the Department of Home Economics, University of Chicago, Chicago.)

(Received for publication, August 2, 1920.)

Through the experiments reported in this paper, it was hoped to determine the effect of cooking on the water-soluble B content of vegetables and to express that effect in a definite quantitative way. Although this latter has not been possible, some significant results have been reached.

Cabbage was used for most of the work, because it is eaten both raw and cooked, and because of its common use among the poor, where it often furnishes one of their best sources of all three vitamines.

Method.

The method used was the yeast growth method as developed by Dr. Roger J. Williams in the Laboratory of Physiological Chemistry of the University of Chicago. In brief, the method consists in examining microscopically hanging drops containing single yeast cells in either a control solution or a solution containing also the substance to be tested for water-soluble B. The number of cells into which the single cells have multiplied in a certain number of hours indicates the presence or absence of the vitamines.

Basal Solution.—The basal solution or control was the same as that used by Miller (1). The control was made up into portions of 25 cc. and these were sterilized for 10 minutes at 15 pounds pressure. They were then kept at room temperature until used.

Sterilization.—The solutions to be tested for water-soluble B were made sterile by the alcohol with which the vitamine had been extracted. That 50 per cent alcohol sterilized the solution was proved in our laboratory since there was no bacterial growth even when the plates were made from a solution which had stood for 3 days. Ordinarily the solutions were tested immediately after being made, but if held over for several days they were kept in the refrigerator.

To secure as nearly sterile conditions as possible, the isolation of the yeast cells, the seeding of the yeast, and the making of the slides were performed in a glass box which was sprayed every few days with alcohol and opened as little as possible to allow work.

Yeast.—The yeast used was the ordinary bakers' yeast (Fleischmann's). It was procured from a local store three times a week and used on 2 days only. Meanwhile it was kept in the refrigerator. It was unwrapped only inside the glass box.

At the time of the experiment, the basal solution, the vitamine solution, and the yeast were taken to the glass box, and the work was performed inside it as follows:

A tiny speck of yeast was removed with a sterile steel pen and suspended in a test-tube containing 10 cc. of sterile water. The suspension was made uniform by blowing into it through a sterile 1 cc. pipette attached to a sterile rubber tube containing a cotton plug. 1 cc. of this yeast suspension was then added by the pipette to the basal solution made up to 30 cc., with sterile water if used as a control, or with the vitamine solution and water to the same volume.

Making of Slides.—As soon as the yeast was added the solution was gently shaken, a sterile pen dipped into it, and 25 very minute drops were made on a cover-slip. These were immediately inverted on hollow slides, and sealed with vaseline. In order to make the drops adhere to the cover-slips, the slips, after being thoroughly cleaned with cleaning solution and distilled water, had been placed in a mixture of benzene and vaseline (1 drop of vaseline to 200 cc. of benzene). They were then wiped with a sterile cloth and dried in the oven before using. Thus a film was left on the slips so that the drops adhered. Two slides at least were made from each solution. The drops were made minute in order that the entire drop might be in the field of vision at one time (with low power), and also to make it more possible to secure drops with only one or two yeast cells. It was impossible to get a suspension in which this was always true but in almost every slide there were at least 8 drops which contained the right number of cells. Not more than six slides were made from one suspension.

Reading of Slides.—When all the slides were made they were examined under the low power of the microscope, and the contents of each drop that contained one or two cells recorded on squared paper, with the relative position of the cells noted, and sometimes the size of the cells also. It was often difficult to recognize cells at this first reading even when the high power was used. After examination the slides were incubated at 30°C. in an electric oven for 18 hours.

They were taken out after 5 or 6 hours and again examined as a check on the first reading. Sometimes what had seemed a single cell at first had grown into such a large colony or such an odd shaped one that it was considered to have grown from a double cell or from the union of two single cells, and was therefore discarded. Sometimes new cells or small colonies were discovered, often near the outer edge of the drop. Usually the single cells had budded or grown to groups of three or four. Occasionally some showed no growth, and sometimes a bit of dirt had been mistaken for a cell. Sometimes the presence of wild yeast was noted at this point.

At the end of 18 hours the final record was taken. Here much judgment was necessary. A single colony or two colonies when their sources could be traced from the beginning gave no difficulty. But when the colonies had broken up into several or changed their position it was hard to feel certain of their source. After seeing two small groups detach themselves from a large one, float off to another part of the drop, and form a new colony normal in appearance, it was felt that not much reliance could be put on the reading in any one drop. Therefore drops showing any unusual appearance were discarded. Sometimes the groups were so large and had grown so much in depth that they could not be counted even under the high power. If they could not be estimated within ten or twenty cells they were discarded. This extremely large growth occurred more often when the single cell in the beginning had been very small, perhaps young, and therefore more vigorous in growth. Drops with wild yeast were of course discarded at this time.

In recording the final results of growth only the growing cells were counted. Williams had recorded also the observed cells which had not grown, but the writer did not feel sufficiently sure of recognizing cells to warrant doing this. Usually results of duplicate slides were averaged and conclusions as to growth drawn from this joint record.

Comparison with Williams' Method.—The above method is practically the same as that of Williams. The chief differences consist in the method of sterilization. Since the effect of heat on the vitamine solution has been the main point in question, it was necessary to extract and sterilize it without heat. This was accomplished by the use of alcohol. Williams sterilized the mixture of control and vitamine solutions in an autoclave. By making up the control in individual portions, this later sterilization was unnecessary, and the yeast suspension could be added directly to the control and solution.

EXPERIMENTAL.

Controls.—During the entire series of experiments, three different mixtures of basal solution were used. These were tested at various times but a control was not run with each experiment. There were many unaccountable variations in the averages of the controls. In general the first two lots averaged 6 to 8, and the third, 10 to 13. Sometimes duplicate slides averaged exactly the same, but more often there was a difference of 2 to 3. The range of growth in the same slide was often about 3 to 12, but sometimes colonies ran as high as 25. In the tables experiments in which the first and second controls were used are combined since they averaged about the same. The third control was used only in the experiments reported in Tables II, V, VI, and VII.

Cabbage.

Small, firm cabbages were procured at a local store and often used for several experiments extending over 2 or 3 weeks. They were kept in the refrigerator and whenever used a fresh cut was made and a portion cut from that, to avoid the part which had been directly exposed to the air.

Raw Cabbage.—A 10 gm. representative portion of cabbage was used; that is, a vertical section was cut so as to include both outer and inner leaves, the central stalk being discarded. It was cut as fine as possible and ground in a mortar to a soft pulp. 10 cc.

of dilute alcohol (50 per cent) were added in which it stood for 15 minutes. It was then transferred to centrifuge tubes, another 10 cc. portion of alcohol was added, and it was centrifuged for 10 minutes.

The liquid was then decanted off and filtered. Another 10 cc. portion of alcohol was added to the residue in the centrifuge tube, thoroughly mixed, and again centrifuged. This process was repeated four times. The residue was then filtered by suction through a Buchner funnel until dry, and this filtrate added to the previous extractions. Thus the vitamine was extracted with five portions of alcohol. To test the completeness of this extraction the residue on the Buchner funnel was soaked in alcohol, centrifuged twice, filtered by suction, and this extraction tested for water-soluble B. The resulting growth was no larger than the average control showing that no vitamine was present; that is, the extraction was as complete as could be made by this method. Since water-soluble B is more soluble in dilute alcohol than in strong (2), this method should be a fairly complete one.

The vitamine solution was made up to (usually) 100 cc. with alcohol. To a control of 25 cc. was added an amount of vitamine solution equivalent to 0.1 gm. of fresh cabbage and the volume made up to 30 cc. The yeast suspension was then added and slides were made and recorded as explained above.

Table I shows the growth in six experiments on raw cabbage including portions from three cabbages. Many preliminary experiments had been made on the raw cabbage with very similar results. The growth from the 61 cells reported here averages 23.4, with a median of 22 and a range from 11 to 80. The range is large, due to one large colony (80) in Experiment 5. Without that, it would have been 11 to 38. In Experiment 4, the cabbage used had been in the refrigerator for several days and was tough and harder to grind to a pulp, which may have interfered with the extraction of the vitamine and so account for the smaller amount of growth.

Boiled Cabbage.[1]—10 gm. of cabbage, cut as above, were cooked for 30 minutes in 100 cc. of boiling water (distilled) in an uncovered beaker. The boiling was made rapid at the end to evaporate as much water as possible. What little water remained was decanted off, filtered, and to it was added the extract from the cabbage. This extract was prepared with alcohol similarly to the raw cabbage.

---

[1] See preceding paper (Miller, E. W., *J. Biol. Chem.*, 1920, xliv, 159) for review of the literature on stability toward heat and alkali.

The second part of Table I shows the results of six experiments with boiled cabbage. The growth of 77 cells averages 26.7 with a median of 24 and a range of 7 to 60. The two parts of Experiments 1, 2, and 3 were performed at the same time under as like conditions as possible. In Experiments 1 and 2 there is almost exact agreement between the averages of the raw and boiled cabbage. This is the more striking in Experiment 1, for here a second extraction had been made (to test the completeness of the first, as explained above) and a like agreement was shown, an average growth of 9.7 for the raw, and 9.6 for the boiled; that is, approximately the same as the control. Certainly the results indicate that the vitamine is not in the least destroyed by boiling for 30 minutes. Several preliminary experiments had been made on steamed cabbage with similar results.

TABLE I.

Growth from 0.1 Gm. of Cabbage, Raw and Boiled.

Experiment No.	Raw.					Boiled.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median	Average.		Minimum.	Maximum.	Median.	Average.
1	14	13	32	26.5	24.7	12	16	40	25	24.6
2	16	11	29	18	19.4	18	7	37	18	19.8
3	12	11	38	23.5	22.8	13	8	30	17	16.3
4	6	12	26	16	17.3					
5	6	15	80	31	36.2					
6	7	16	33	26	25.1					
7						11	20	48	38	36.8
8						10	7	55	25.5	26.6
9						13	12	60	35	34.4
Total.	61	11	80	22	23.4	77	7	60	24	26.7

Table II shows two experiments in which portions were boiled for 30 and 60 minutes, the two parts of the experiment being carried on at the same time. The average growth for the two parts of the experiments is fairly close, 42.5 for the 30 minute cabbage, and 44.5 for the 60 minute. The averages for the separate experiments, however, are somewhat different. The range in each experiment is also large. (This 30 minute cabbage is not included among the experiments in Table I, because the higher control was used.)

Boiled with Acid.—In two experiments the cabbage was boiled with about 5 cc. of vinegar added to the 100 cc. of water. The resulting growth was very great. The colonies were not only larger than usual but often were broken up into several and were uncountable. Some new colonies appeared where no cells were recognized on the 5 hour reading. Certainly water-soluble B is not destroyed by boiling with acid. This is in agreement with Williams (3) and with McCollum and Simmonds (4) who find only slight destruction, if any, with nitrous acid or hydrochloric acid.

TABLE II.

Growth from 0.1 Gm. of Cabbage, Boiled 30 and 60 Min.

Experiment No.	30 min.					60 min.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.		Minimum.	Maximum.	Median.	Average.
1	8	12	100	34	40	17	12	140	40	54
2	15	18	100	36	44	12	15	100	34	38
Total.	23	12	100	36	42.5	27	12	140	36	44.5

Boiled with Alkali.—Table III shows the results of three experiments in which the 10 gm. portion of cabbage was boiled in 100 cc. of water to which 0.1 gm. of baking soda was added. The 41 cells show an average growth of 24.1, a median of 21, and a range of 8 to 70. The actual growth, however, was much larger as some broken or uncountable colonies were discarded.

These results with acid and alkali are in agreement with Williams (3) who found that "this material when acted on by acid or alkali is changed (presumably by hydrolysis) to a form which is more immediately available for the yeast."

Loss in Cooking Water.—In this series of six experiments 10 gm. portions of cabbage were boiled in the usual way for 30 minutes. The cooking water, however, was decanted off, mixed with alcohol, and tested separately. The vitamine in the drained cabbage was extracted in the usual way and tested. In testing the cooking water and the cabbage extract amounts equivalent to 0.1 gm. of raw cabbage were used as usual.

TABLE III.

Growth from 0.1 Gm. of Cabbage, Boiled with $NaHCO_3$.

Experiment No.	No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.
1	10	10	70	16	31
2	15	8	42	25	25.1
3	16	9	30	21	18.9
Total........	41	8	70	21	24.1

TABLE IV.

Growth from Cooking Water and Drained Cabbage.

Experiment No.	Cooking water.					Drained cabbage.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.		Minimum.	Maximum.	Median.	Average.
1	11	6	60	22	28.1	12	10	60	25.5	30
2	16	9	75	24.5	28.4	10	10	70	21.5	28.6
3	9	10	42	20	23.3	15	7	32	14	15.7
4	9	7	36	30	25.4	9	8	24	18	18.1
5	14	16	29	23	22.9	7	15	29	20	19.7
6	3	28	40	29	32.3	8	21	49	29	32.6
Total.	62	6	75	24	26.1	61	7	70	22	23.6

Table IV summarizes the results and shows that in three of the experiments the growth from cooking water and from extract from drained cabbage was practically identical—half the vitamine in each. In the other three, the cooking water contained a trifle more than half. Considering the six experiments together, the 62 cells from the cooking water average 26.1 with a median of 24, and the cells from the drained cabbage extract average 23.6, with a median of 22. The ranges are very close, 6 to 75 in one case and 7 to 70 in the other.

The combined growth from 0.1 gm. of drained cabbage and its cooking water should equal the growth from 0.1 gm. of boiled cabbage with the water retained (as in Table I). This is nearly true in Experiment 3 in Table IV and Experiment 9 in Table I. These two portions of cabbage were cooked at the same time and the experiments carried out under as like conditions as possible. The control used with these solutions averaged about 8. Allowing for this control the increased growth from the boiled cabbage with the water retained was 34.4 − 8 = 26.4 (Table I, Experiment 9), the growth from the cooking water 23.3 − 8 = 15.3, and from the corresponding extract 15.7 − 8 = 7.7 or a total of 23 from 0.1 gm. of cabbage (Table IV, Experiment 3). These averages, 26.4 and 23, are fairly near.

This series of experiments shows that about half the vitamine is lost in the cooking water. In actual household practice where larger pieces of the vegetable are cooked and therefore less surface is exposed less vitamine would be dissolved out in the cooking water. This retention of more than one-half the vitamine, especially in strongly flavored vegetables such as cabbages and onions (Table VII) where the cooking water is usually discarded, is very important. As far as can be ascertained this is the first attempt to discover the proportion of water-soluble B lost in the cooking water.

Daniels and McClurg (5) warn against discarding water around cooked or canned vegetables because their rats grew on the liquor from cooked beans. But they were dealing with an extraction rather than ordinary "cooking water" since the cooked material was comminuted by pressing through a strainer, the liquor filtered off, and the beans were washed several times with water. In the experiments of Daniels and Byfield (6), in adding water-soluble B to the food of artificially fed babies in order to secure proper growth they used both the alcoholic extract of carrots, turnips, and celery, and a soup made from the same vegetables. Since the water extract was nearly as effective as the alcoholic extract it shows that much of the vitamine had been extracted.

Onions.

A few experiments were made on onions. They were cut and treated similarly to the cabbage and the same amounts were used.

Raw Onions.—In two experiments on raw onions (Table V) the average growth from 0.1 gm. of Silver Skins was 35.2, and of Spanish onions 26.1. Of course, this one experiment indicates little as to the relative value of the two kinds but simply shows the presence of water-soluble B in both.

TABLE V.

Growth from 0.1 Gm. of Raw Onion.

Kind.	No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.
Silver Skins...........	20	20	55	35.0	35.2
Spanish..............	16	15	50	25.5	26.1

TABLE VI.

Growth from 0.1 Gm. of Boiled Onion.

Kind.	No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.
Spanish..............	11	11	28	23	21.5
"	4	16	50	41.5	37.2
Total.................	15	11	50	25	25.7

Boiled Onions.—Two experiments on boiled Spanish onions (Table VI) showed an average growth of 25.7, as against 26.1 for the raw Spanish onions. The heating had not destroyed the vitamine.

Loss in Cooking Water.—Two experiments comparing the cooking water with the extract from the boiled onion (Table VII) give the same results as cabbage; namely, that only about one-half the vitamine is lost in the cooking water.

No attempt should be made here to compare the vitamine content of cabbage and of onion. The experiments with onions are too few, and the results in both not sufficiently consistent. However, the presence of water-soluble B in onions is here shown. This is important in view of the apparent contradiction (7) between the work of Osborne and Mendel (8) who proved its presence by the growth of rats, and of McCarrison (9) whose pigeons, fed on polished rice, butter fat as a source of fat-soluble A, and onions as a source of C, developed polyneuritis even more quickly than those on a diet of polished rice alone. If the antineuritic, antiberiberi vitamine is not identical with the growth-promoting vitamine necessary for rats, as has not yet been definitely proved (10), although generally so considered, these results would show the yeast growth vitamine to be identical with the "growth-promoting" vitamine.

TABLE VII.

Growth from Cooking Water and Drained Onion.

Experiment No.	Cooking water.					Drained onion.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.		Minimum.	Maximum.	Median.	Average.
1	22	18	43	35	33.1	14	18	52	35	31.7
2	9	12	50	30	29	15	12	40	25	25.5
Total.	31	12	50	30	31.9	29	12	52	30	28.3

DISCUSSION.

As a strictly quantitative test the method has proved somewhat disappointing. Although duplicate slides and duplicate experiments have sometimes been remarkably consistent in range as well as in average even here the range within the slide has been large. Within the same drop even, 1 cell may grow to 20 and another to 40.

It would seem that the differences in growth of the cells must often be due to a difference in the individual yeast cells themselves. Sometimes they varied in shape and appearance as has been noted. Then, too, there may be some difference in the yeast cakes. Other substances may be introduced with the yeast cells.

The possible errors in reading the slides have already been noted, and most of them have been recognized by Williams.

Among other factors which may have affected the results, in spite of all efforts to standardize the method, may be included: variations in the cabbage—different cabbages, parts of the same cabbage, changes due to keeping; variations in the extract, completeness, time before using; influence of other substances which were probably extracted with the vitamine, such as mineral salts, nitrogenous substances, and carbohydrates; variations in control; and variations in temperature of the glass box which was sometimes much above room temperature.

CONCLUSIONS.

Although strictly quantitative results have not been secured by these experiments certain definite points have been made.

1. Water-soluble B in cabbage is not destroyed by boiling for 30 or 60 minutes.
2. It is not destroyed by boiling with acid or alkali.
3. It is present in onions and is not destroyed by boiling.
4. Not more than one-half the water-soluble B is lost in the cooking water from cabbage or onions boiled for 30 minutes.

I wish to express my thanks to Dr. F. C. Koch, Dr. R. J. Williams, and Miss E. W. Miller for helpful suggestions concerning this method as used in their laboratory.

BIBLIOGRAPHY.

1. Miller, E. W., The effect of cooking on the water-soluble vitamine in carrots and navy beans, *J. Biol. Chem.*, 1920, xliv, 159.
2. Osborne, T. B., Wakeman, A. J., and Ferry, E. L., Preparation of protein free from water-soluble vitamine, *J. Biol. Chem.*, 1919, xxxix, 35.
3. Williams, R. J., The vitamine requirement of yeast, *J. Biol. Chem.*, 1919, xxxviii, 465.
4. McCollum, E. V., and Simmonds, N., A study of the dietary essential, water-soluble B, in relation to its solubility and stability towards reagents, *J. Biol. Chem.*, 1918, xxxiii, 55.
5. Daniels, A. L., and McClurg, N. I., Influence of high temperatures and dilute alkalies on the antineuritic properties of foods, *J. Biol. Chem.*, 1919, xxxvii, 201.

6. Daniels, A. L., and Byfield, A. H., The rôle of the antineuritic vitamin in the artificial feeding of infants, *Am. J. Dis. Child.*, 1919, xviii, 546.
7. Blunt, K., and Wang, C. C., The present status of vitamines, *J. Home Econ.*, 1920, xii, 1.
8. Osborne, T. B., and Mendel, L. B., Nutritive factors in plant tissue, *J. Biol. Chem.*, 1919, xxxix, 29.
9. McCarrison, R., The pathogenesis of deficiency disease, *Indian J. Med. Research*, 1919, vi, 275, 550.
10. Mitchell, H. H., On the identity of the water-soluble growth-promoting vitamine and the antineuritic vitamine, *J. Biol. Chem.*, 1919, xl, 399.

CAN "HOME GROWN RATIONS" SUPPLY PROTEINS OF ADEQUATE QUALITY AND QUANTITY FOR HIGH MILK PRODUCTION? II.*

By E. B. HART and G. C. HUMPHREY.

With the Cooperation of S. Lepkovsky.

(From the Departments of Agricultural Chemistry and Animal Husbandry, University of Wisconsin, Madison.)

(Received for publication, August 5, 1920.)

In 1919[1] we published results which showed that it was impossible to maintain nitrogen equilibrium in dairy cows of high daily milk production with a ration made from a cereal grain, *red clover hay*, and corn silage. The protein content of such a ration was inadequate in quantity or quality for continuous high production without imposing a considerable draft on the cow's protein tissues. We showed that with clover hay as the roughage in the above ration a high milk-producing cow would not only fall off in the volume of milk secreted more rapidly than normally occurs with the progress of lactation, but that she would show a negative nitrogen balance. On the other hand it was shown that it was possible, with cows of moderate milking capacity of 20 to 25 pounds of milk daily containing 14 per cent of solids, but with large feed consumption, to maintain milk flow as well as nitrogen equilibrium over a period of at least 4 months on a ration made of red clover hay, corn silage, and a cereal grain.

We expressed the view that there was a possibility that through further research a combination of "home grown" materials could be found that would be more effective with respect to quantity or quality of proteins than the ration used in our earlier experiments.

---

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1919, xxxviii, 515.

Naturally the very great prominence given to *alfalfa* as a forage plant in all northern areas of this country led us to use it in substitution for red clover hay as a possible material of either greater protein efficiency or higher protein content. The experiments to be described in this paper are based on the use of alfalfa hay, together with corn silage and a cereal grain as the ration. The grains used were corn, barley, oats, or a mixture of the three. As in all work of this kind, metabolism experiments are absolutely necessary if we are to have positive knowledge in regard to the intake and outgo of nitrogen. Without such data no definite information of value on the status of nitrogen metabolism in the animal can be accumulated, especially when short periods of time are involved.

Much to our surprise we found it possible not only to maintain nitrogen equilibrium in cows milking 40 to 45 pounds of 11 per cent milk daily, but to maintain that flow over a period of 16 weeks with a ration made from alfalfa hay, a cereal grain, and corn silage. The alfalfa used was not specially selected, but was taken from a car lot bought by the University as a commercial product and grown in the West. It contained 15.5 per cent of protein (N × 6.25) which is but 0.6 per cent higher than the average of 250 analyses of this plant as given by Henry and Morrison.[2]

It is true, however, that most of the materials used in the construction of this alfalfa ration were appreciably higher in nitrogen than the materials used in our clover hay ration of the preceding year; but the main difference between the two rations was the higher level of crude protein secured in this ration and made possible by the use of alfalfa. Data showing the differences in nitrogen content of the materials used in the clover ration of 1918–19 and the alfalfa ration of 1919–20 are given in Table I. With the exception of corn silage, the data are based on the air-dried material.

From our metabolism data it does not appear that a unit of nitrogen absorbed from the clover-silage-cereal grain ration was less productive of milk and tissue protein than a unit of nitrogen absorbed from the alfalfa-silage-cereal grain ration. On the basis of weekly periods there was approximately 0.45 to 0.64 gm. of nitrogen elaborated in product per gm. of absorbed nitrogen on the clover ration, and 0.41 to 0.59 gm. per gm. of absorbed nitrogen on the alfalfa ration. These differences between the effectiveness of the two rations are too slight to be given any significance and should not be accepted as indicating that we had a quality of protein in the alfalfa ration superior to that of the clover ration. The greater efficiency of the alfalfa ration over the clover ration is probably to be attributed to the higher plane of protein intake made possible by the higher nitrogen content of the alfalfa ration. The alfalfa hay itself was mainly responsible for this higher nitrogen level.

---

[2] Henry, W. A., and Morrison, F. B., Feeds and feeding, Madison, 15th edition, 1915, 640.

TABLE I.

Nitrogen Content of Feeds.

Material.	Nitrogen.	
	1918-19.	1919-20.
	per cent	*per cent*
Corn grain	1.43	1.65
Oat "	1.48	1.70
Barley "	1.80	1.89
Clover hay	1.74	
Alfalfa "		2.48
Corn silage	0.36	0.45

EXPERIMENTAL.

Our plan was to use a ration of corn silage and alfalfa hay to which ground corn was added in the first period of 4 weeks, ground oats in the second period of 4 weeks, ground barley in the third period of 4 weeks, and a mixture of approximately equal parts of the three grains in the fourth period of 4 weeks. The alfalfa hay was cut before feeding. This was done in an ordinary feed cutter. The grains were ground. Provision for a constant energy intake with the different rations was made through the use of corn-starch. In some cases the amount of grain allowed was above, while in other cases it was below, the proportion of 1 pound of grain to 3 pounds of milk—a practical rule often used and recommended by animal husbandry teachers. The animals were fed all that they would consume but their consumption did not conform closely with the energy needs as set forth in the Armsby standards. For example, Cow 1 consumed 58 pounds of the corn ration which provided 20 therms. On the basis of her milk production (42 pounds of 3 per cent (fat) milk daily) and live weight (1,200 pounds) she should have received 16 therms. Cow 2 consumed 50 pounds of the corn grain ration or its equivalent as dictated by appetite. This provided an intake of 17.2 therms. Her energy requirement as formulated by the Armsby standard based on milk production (30 pounds daily of 3.5 per cent milk) and live weight (1,200 pounds) would have been 14.1 therms.

Our plan had been to use three animals for the four periods of 4 weeks each, but we were not successful in carrying all of them through the entire time. We succeeded in our plan with three of the animals; but No. 3 went off feed at the end of 4 weeks with the necessity of providing a fourth animal for the remaining three periods.

One of the animals used was a pure bred Holstein, the other three were grade Guernseys. They were fed and milked twice daily, with quantitative collection of all excreta. The urine and feces were analyzed daily, while a 7 day composite of the milk was collected for analysis. The composition of the rations fed is shown in Table II. Data are given for 50 pounds of the corn grain ration and its equivalent in protein and therms in the case of the oat, barley, and mixed grain rations. It should be remembered that 50 pounds or its equivalent of any particular ration was not necessarily fed, but that portion allowed which was entirely consumed.

A nutritive ratio of 1:7.9 was made possible with the materials used. The cows differed in their consumption, some eating more than 50 pounds of the ration while others ate less. Cow 1, a Holstein, consumed 58 to 60 pounds of the ration daily, while Cow 3, a smaller animal and producing less milk, consumed 46 to 48 pounds of the ration. In 50 pounds of this ration there were provided 3.44 pounds of *crude* protein. From our records of nitrogen metabolism about 60 per cent of the nitrogen was digested (absorbed). This amounts to a provision of 2.06 pounds of *digestible crude* protein with 50 pounds of the ration. This amount of protein was ample over the period of observation for

Cow 2, producing daily 30 pounds of 3.2 per cent milk (fat) and weighing 1,200 pounds. The Armsby standard would require 1.98 pounds of digestible *true* protein daily for similar performance and with no provision for the influence of the quality of

TABLE II.

Composition of Ration.

	Weight.	Total N.	Therms.	Total protein.
Corn grain ration.				
	lbs.	*gm.*		*lbs.*
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Grain	10	74.9	8.88	
Starch				
Total	50	250.0	17.28	3.44
Oat grain ration.				
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Grain	9.7	74.9	6.62	
Starch	2.2		2.20	
Total	51.9	250.0	17.22	3.44
Barley grain ration.				
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Grain	8.7	74.9	7.02	
Starch	1.8		1.80	
Total	50.5	250.0	17.22	3.44
Mixed grain ration.				
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Corn	3.3	74.9	7.52	
Oats	3.2			
Barley	2.9			
Starch	1.3		1.30	
Total	50.7	250.0	17.22	3.44

the protein. On the basis of our analysis of the materials used and the coefficients of digestibility given in the text-books on feeding, 50 pounds of our ration would have provided 2.28 pounds of *digestible crude* protein. This is not in very close agreement with the Armsby standard, if the amount of digestible crude protein supplied by our ration is reduced to *digestible true* protein; in fact it would fall considerably below the provision of the Armsby standard; but there probably can be no question but that the free amino or simple peptide nitrogen of the corn silage and alfalfa hay is of direct nutritive value, although it may not be precipitated by Stutzer's[3] reagent which is used in differentiating crude from true protein. The Wolff-Lehmann standard would require 3.3 pounds of *digestible crude protein* for a 1,000 pound cow, giving 27.5 pounds of milk, while modified standards suggested by Henry and Morrison would require 2.3 pounds of *digestible crude protein* for a 1,000 pound cow giving 30 pounds of 3.5 per cent milk.

In three of the periods extra energy and under practical conditions probably unnecessary energy was provided in the form of corn-starch. This was done to make the oat, barley, and mixed grain rations as high in net energy as the corn grain ration, which, with its corn-meal, contained a higher therm value than the other rations. We set our standard as the corn grain-alfalfa-silage mixture in order to get as much protein into the ration as possible, necessitating thereby an equal therm provision in all the other rations. It can be seen from Table II that we could have omitted from the oat, barley, or mixed grain ration the added energy in corn-starch and still have provided adequate therms and protein.

The records of nitrogen metabolism are shown in Tables III to V. Positive nitrogen balances were maintained with all the animals. The records for Cows 1, 3, and 4 would indicate some protein storage. Cow 2 was nearer nitrogen equilibrium, although the greater part of the time she was in a distinctly positive nitrogen balance. All these animals had been bred in the first 2 weeks of confinement to experimental conditions and part of the nitrogen storage is to be correlated with fetus building. The live weights of these animals were well maintained, although no great importance is to be attached to a maintenance of live weight as indicating a positive nitrogen balance, particularly over a period of 3 to 4 months with a cow.

---

[3] Stutzer, N., in Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii, 477.

TABLE III.

Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.					Milk per week.
	Intake.	Feces.	Urine.	Milk.	Balance.	
	Corn grain ration, Animal 1.					
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 5-11, 1919......	1,910.7	699.0	519.0	554.6	+138.1	305
" 12-18, 1919......	2,030.7	719.2	515.6	559.8	+236.1	301
" 19-25, 1919......	2,030.7	824.8	493.2	528.3	+184.4	284
" 26, 1919-Jan. 1, 1920..........	2,030.7	780.4	520.8	576.8	+152.7	291
	Oat grain ration, Animal 1.					
1920						
Jan. 2- 8............	2,025.8	761.5	492.7	582.1	+189.5	290
" 9-15............	2,025.8	743.2	558.6	568.4	+155.6	288
" 16-22............	2,025.8	733.0	572.9	538.3	+181.6	285
" 23-29............	2,025.8	757.6	592.6	559.9	+115.7	293
	Barley grain ration, Animal 1.					
Jan. 30-Feb. 5.......	2,025.8	771.6	562.9	568.6	+122.7	303
Feb. 6-12...........	2,025.8	717.5	549.7	562.6	+196.0	292
" 13-19...........	2,025.8	836.6	461.5	582.6	+145.1	288
" 20-26...........	2,025.8	796.9	458.1	561.4	+209.4	290
	Mixed grain ration, Animal 1.					
Feb. 27-Mar. 4.......	2,025.8	798.3	429.7	557.9	+239.9	294
Mar. 5-11...........	2,025.8	760.0	465.7	568.3	+231.8	294
" 12-18...........	2,025.8	782.9	457.5	559.8	+225.6	287
" 19-25...........	2,025.8	800.4	480.7	568.2	+176.5	290

The initial and final live weights of these animals were as follows: No. 1, 1,174 to 1,250; No. 2, 1,220 to 1,188; No. 3, 1,017 to 1,007; No. 4, 1,117 to 1,108 pounds.

The milk production, both in composition and flow, was maintained. It is exactly on this point that our data c give a final answer to the question whether the milk flow milk composition would have been maintained over an

TABLE IV.

Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.				
	Intake.	Feces.	Urine.	Milk.	Balance.
			Corn grain ration, Animal 2.		
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Dec. 5–11, 1919......	1,751.4	573.0	607.4	461.1	+109.9
" 12–18, 1919......	1,751.4	641.6	677.7	445.8	−13.7
" 19–25, 1919......	1,751.4	620.0	586.5	437.1	+107.8
" 26, 1919–Jan. 1, 1920..........	1,751.4	611.0	705.9	442.8	−8.3
			Oat grain ration, Animal 2.		
1920					
Jan. 2–8............	1,750.0	619.2	626.2	436.2	+68.4
" 9–15............	1,750.0	645.7	648.1	433.3	+22.9
" 16–22............	1,750.0	577.0	714.9	402.3	+55.8
" 23–29............	1,750.0	752.2	630.8	443.9	−76.9
			Barley grain ration, Animal 2.		
Jan. 30–Feb. 5.......	1,750.0	676.5	597.9	433.4	+42.2
Feb. 6–12...........	1,750.0	621.8	583.0	417.0	+128.2
" 13–19...........	1,750.0	677.0	645.2	416.1	+11.7
" 20–26...........	1,750.0	622.3	551.1	423.6	+153.0
			Mixed grain ration, Animal 2.		
Feb. 27–Mar. 4.......	1,750.0	647.8	564.6	416.8	+120.8
Mar. 5–11...........	1,750.0	628.8	598.7	422.7	+99.8
" 12–18...........	1,750.0	680.4	620.9	420.1	+28.6
" 19–25...........	1,750.0	674.9	632.7	419.9	+22.5

gestation period by a ration providing no more protein tha one we used. We can only answer the question for a peri 4 months. Over that time the volume of milk secreted, as as its composition, was well maintained. Only that shri in volume incident to a progressive lactation was observed. The composition of the milk and average daily production for a single week in each feeding period are given in Table VI. Charts 1 to 3 are added to show the maintenance of nitrogen equilibrium as well as the constancy in milk nitrogen elaboration over the 16 weeks of observation.

TABLE V.

Record of Nitrogen Balance, Milk Production, Etc.

Date.	Nitrogen.					Milk per week.
	Intake.	Feces.	Urine.	Milk.	Balance.	
	Corn grain ration, Animal 3.					
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 5–11, 1919	1,610.7	612.1	479.6	435.8	+83.2	178
" 12–18, 1919	1,610.7	566.4	444.4	458.2	+141.7	180
" 19–25, 1919	1,610.7	627.1	397.3	448.0	+138.3	170
" 26, 1919–Jan. 1, 1920	Off feed.					
	Oat grain ration, Animal 4.					
1920						
Jan. 2– 8	1,610.0	605.4	488.5	501.5	+14.6	217
" 9–15	1,610.0	504.0	462.4	507.7	+135.9	217
" 16–22	1,610.0	588.3	489.2	463.3	+69.2	195
" 23–29	1,610.0	591.1	485.4	484.0	+49.5	212
	Barley grain ration, Animal 4.					
Jan. 30–Feb. 5	1,610.0	606.3	503.3	493.5	+6.9	211
Feb. 6–12	1,610.0	528.6	502.2	481.1	+98.1	201
" 13–19	1,610.0	569.7	453.7	465.5	+121.1	188
" 20–26	1,610.0	551.7	461.3	459.6	+137.4	188
	Mixed grain ration, Animal 4.					
Feb. 27–Mar. 4	1,610.0	563.0	444.0	447.4	+155.6	189
Mar. 5–11	1,610.0	636.4	420.2	460.2	+93.2	194
" 12–18	1,610.0	640.2	439.7	458.7	+71.4	194
" 19–26	1,610.0	630.1	450.2	455.9	+73.8	192

In our earlier paper reviewing our experience with clover hay we stated that "from what we know of the physiology of milk secretion and the relation of protein to that function it would appear to be unprofitable not to supply protein in quantities sufficient to maintain nitrogen equilibrium in a milking animal." We believe that to be an axiom of good dairying, but we must also insist that the set nutritive ratio for milk production is as fallacious, scientifically, as the set nutritive ratio for growth. In the light of modern protein chemistry our knowledge of the protein requirements of dairy cows is in as confused a state as our knowledge of the protein requirements for human nutrition.

TABLE VI.

Composition of Milk and Average Daily Flow in a Selected Week of Each Period.

	Animal 1.			
	Dec. 18.	Jan. 15.	Feb. 12.	Mar. 11.
Total solids, *per cent*	11.40	11.50	11.40	11.30
Fat, *per cent*	3.00	3.00	3.00	3.00
Nitrogen, *per cent*	0.41	0.43	0.42	0.43
Milk daily, *lbs*	43.00	41.10	41.70	42.00
	Animal 2.			
Total solids, *per cent*	11.40	11.40	11.90	11.70
Fat, *per cent*	3.20	3.20	3.60	3.50
Nitrogen, *per cent*	0.40	0.40	0.43	0.44
Milk daily, *lbs*	34.50	30.70	29.80	30.10
	Animal 4.			
Total solids, *per cent*		13.40	13.65	14.00
Fat, *per cent*		4.40	4.80	5.00
Nitrogen, *per cent*		0.51	0.51	0.52
Milk daily, *lbs*		30.00	28.70	27.70

There are advocates of high levels as well as advocates of more moderate levels. The guiding facts will only come to light when quality and quantity of protein are involved in experimental inquiry, through the use of metabolism experiments. In the present state of our knowledge a nutritive ratio set at a high level becomes safe practically, and that of course justifies its use until we have positive evidence that high levels of protein are injurious or unnecessary with certain specific mixtures.

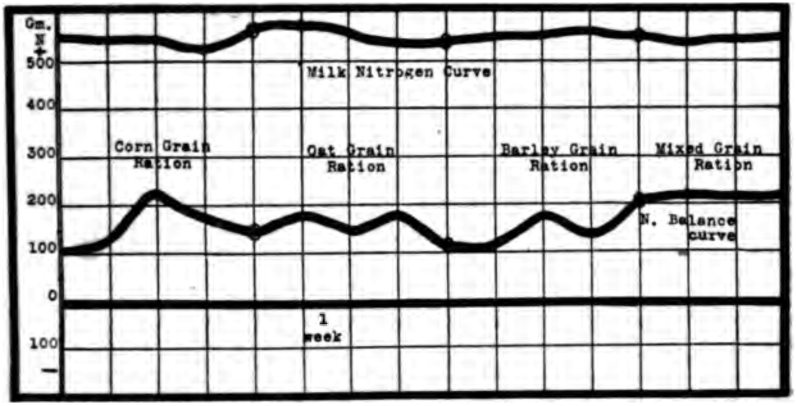


Chart 1. Animal 1, showing nitrogen balance and milk nitrogen produced. A positive nitrogen balance as well as a constant amount of milk nitrogen was maintained for 16 weeks.

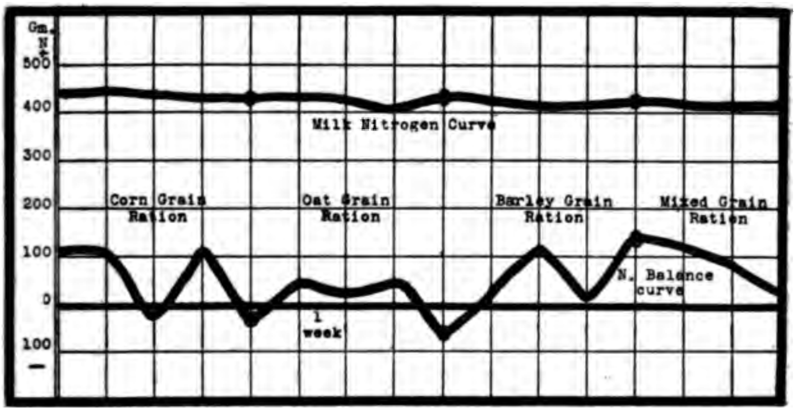


Chart 2. Animal 2, showing nitrogen balance and milk nitrogen produced. Nitrogen equilibrium as well as a constant amount of milk nitrogen was maintained for 16 weeks.

In the experiments reported here it is evident that we have in a cereal grain-corn silage-*alfalfa* ration a "specific mixture" which can, when the nitrogen content is comparable to the nitrogen content of the materials used in this work, maintain nitrogen equilibrium and the milk flow of comparatively high producing cows over a period of at least 16 weeks. It should, of course, be determined whether such milk production as here experienced could be maintained over a full lactation period, with only that shrinkage in flow incident to an advancing lactation, before definite advice to dairymen is given. Haecker[4] in his long observations on the nutrition of dairy cows found that in the earlier years a low protein ration appeared as effective as the higher one, but toward the end of 3 years the low protein group showed deficient vitality, apparently indicating a lack of protein. However, the nutritive ratio used by Haecker was a wider one than used in our experiments. It should also be made perfectly clear that our ration involved the use of a cereal grain in addition to corn silage and alfalfa and was not made up from the two latter materials alone. Further, we could observe no differences in the nutritive efficiency of our rations whether the grains used were corn, oats, barley, or a mixture of the three.

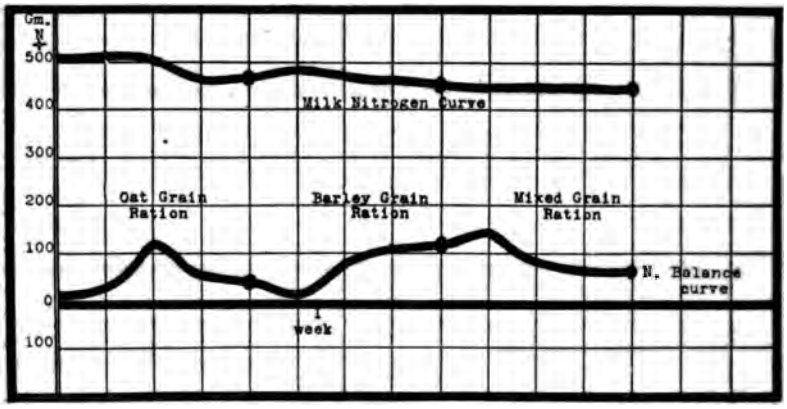


CHART 3. Animal 4, showing nitrogen balance and milk nitrogen produced. A positive nitrogen balance was maintained, with but a slight decline in the milk nitrogen produced over a period of 12 weeks.

---

[4] Haecker, T. L., *Minnesota Exp. Station, Bull. 71*, 1901; *Bull. 79*, 1903; *Bull. 140*, 1914.

SUMMARY.

1. In this paper are presented data which show that it is possible to furnish dairy cows of high milk-producing capacity with a protein level of sufficient magnitude to maintain that capacity for 16 weeks from an alfalfa-corn silage-cereal grain mixture, the latter being corn, barley, oats, or a mixture of the three.

2. Four cows were used in this work. No. 1 produced 40 to 45 pounds of 11.4 per cent (solids) milk daily over a period of 16 weeks, on this "home grown" ration, maintaining nitrogen equilibrium as well as a sustained milk flow and milk composition. The other three animals were not so large producers as No. 1, but with less ration maintained a positive nitrogen balance and sustained milk flow and milk composition over the period of observation.

3. This ration had a nutritive ratio of 1:7.9 and provided less *digestible true* protein than the requirements of the Armsby standard. It provided somewhat less *digestible crude* protein than required by the modified standards suggested by Henry and Morrison. Longer observations must decide whether such a ration is adequate for an entire lactation period or for the practical period of barn feeding, unsupplemented by pasture grasses.

NOTE ON THE PRESERVATION OF SPECIMENS OF BLOOD INTENDED FOR BLOOD SUGAR DETERMINATIONS.

By W. DENIS and MARTHA ALDRICH.

From the Chemical Laboratory, Massachusetts General Hospital, Boston.)

(Received for publication, July 16, 1920.)

The relatively rapid decrease in the sugar content of blood, even when preserved at low temperatures, is too well known to require comment.

Both from the standpoint of the biochemist and of the clinical worker there is great need of some simple method whereby samples of blood intended for sugar determinations might be preserved, if necessary for several days, until some suitable time or place may be found in which the analyses can be made. This need is particularly acute in hospital work, and we have, therefore, experimented with a number of substances, which would be expected, theoretically at least, to limit enzyme action and at the same time to introduce no errors in the subsequent determination of blood sugar by the revised method of Folin and Wu,[1] the procedure used as a routine in this laboratory.

We believe that we have found a suitable preservative in formaldehyde, which we use in the proportion of 1 drop of commercial "formalin" (containing 40 per cent formaldehyde) to 5 cc. of oxalated blood. The dropper employed gives 30 drops per cubic centimeter. The addition of a larger amount of formaldehyde (3 drops to 5 cc. of blood) adds no advantage, nor apparently does it introduce any error.

We are aware of the fact that formaldehyde is ordinarily classed as a weak reducing agent, and that the advisability of its use in this connection may be questioned on theoretical grounds. In fact, we only tried it after we had exhausted every other possibility. We have, however, repeatedly demonstrated the fact that formaldehyde in amounts ten times greater than used in the blood sugar determinations produces no formation of cuprous oxide in the alkaline copper tartrate solution used in the Folin-Wu procedure, a fact probably due to the extremely low degree of alkalinity possessed by this reagent.

---

[1] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

The following experimental data is cited in proof of the above statements.

Blood No. 12.

Amount of blood sugar per 100 cc.	Amount of formaldehyde.
mg.	*drops*
99.	.None.
100.	1
100.	2
100.	3

Blood No. 16.

Amount of blood sugar per 100 cc.	Amount of formaldehyde.
mg.	*drops*
101.	None.
102.	1
101.	2
101.	3

It will be found that if to 2 cc. of the tungstic acid filtrate there is added 1 drop of formaldehyde, and the sugar determination is made on this portion of filtrate in the usual way, results will be secured which are identical with those obtained in portions of the same filtrate to which no formaldehyde has been added.

By means of formaldehyde used in the manner above described we have been able to prevent glycolysis in blood for as long as 96 hours at temperatures varying from 20–33°C. It seems probable that this period might be greatly extended, but as 4 days appeared to cover all ordinary needs we have not extended our experiments to cover a longer period of time.

We have also made a few observations to determine whether the formaldehyde-containing filtrate can be used for the determinations of the various non-protein blood constituents, such as non-protein nitrogen, urea, creatinine, and uric acid. While

TABLE I.

Experimental Results Showing the Effect of Formaldehyde in Preventing Glycolysis in Non-Diabetic Blood.

Sample No.	Amount of sugar per 100 cc. of blood.			
	20 to 60 min. after bleeding.	After 24 hrs.	After 36 hrs.	After 72 hrs.
	mg.	*mg.*	*mg.*	*mg.*
2	98	100		
3	103	107		
4	91	92		
1	95		95	
5	100		100	
6	90		94	
12	99			100
9	138			138
13	116			119
17	82			82
15	100			100

TABLE II.

Experimental Results Showing the Effect of Formaldehyde in Preventing Glycolysis in Diabetic Blood.

Sample No.	Amount of sugar per 100 cc. of blood.		
	20 to 60 min. after bleeding.	After 48 hrs.	After 96 hrs.
	mg.	*mg.*	*mg.*
22	210		214
23	125	125	
24	158	161	
25	156	162	
26	128	121	133
27	196	186	200
29	232	232	232
30	188	184	190
31	146	143	150
32	143	145	
33	174	178	
34	154	160	
35	202	208	
37	121	122	

we have been able to obtain good results in the determination of these two latter constituents, the values obtained for non-protein nitrogen and for urea (determined by the urease method) are invariably low.

The results presented in Tables I and II indicate the possibilities of this method of preservation. The samples of non-diabetic blood were taken in the afternoon soon after the midday meal; all the samples of diabetic blood were obtained from fasting subjects.[2]

[2] For the diabetic material we are indebted to Dr. Elliott P. Joslin.

AN APPARATUS FOR CONTINUOUS DIALYSIS OR EXTRACTION.*

By HUBERT MANN.

(*From the Chemical Laboratory, Montefiore Home and Hospital, New York.*)

(Received for publication, August 19, 1920.)

The apparatus was constructed during the course of some work in which it was necessary to carry out dialysis under reduced pressure.

The apparatus consists essentially of a flask, a condenser, and a vessel so arranged that the system may be evacuated and that liquid placed in the apparatus may be caused to circulate by means of continuous evaporation and condensation. Substances that will pass into solution are extracted and concentrated under thermal and chemical conditions favorable for the preservation of labile and easily decomposed compounds.

Fig. 1 is a drawing of the apparatus. The flask, *F*, is equipped with a ground joint, *J*, provided with a mercury cup. *H* is a heating unit suitable for heating the flask, *F*. An incandescent bulb may be used. *s* is a stop-cock attached to a short tube. *T* is a condenser tube and *C* is the condenser. *V* is a vessel fitted with a ground stopper, *S*, provided with a mercury cup. *B* is a bag of collodion, cloth, or other material suspended in the vessel, *V*. *t* is a narrow tube through which fluid in *V* can flow back into *F*.

The method of operation is as follows:

1. The substance to be extracted or dialyzed is placed in the bag, *B*, which is suspended in the vessel, *V*.
2. Enough of the desired solvent is introduced into the vessel, *V*, so that it overflows through the tube, *t*, into the flask, *F*, partly filling the flask.

---

* The expenses of this work were defrayed from a fund founded by the late Dr. H. S. Oppenheimer.

3. The apparatus is then exhausted to the desired degree through the tube which is fitted with the stop-cock, *s*, and this stop-cock is then closed.

4. The condenser tube, *T*, is now cooled by means of a stream of water circulating through the condenser, *C*, and the flask, *F*, is warmed by the heating unit, *H*.

There results a continuous evaporation of the fluid in the flask, *F*, and a continuous condensation of this evaporated fluid in the condenser tube, *T*. The condensed liquid flows into the vessel, *V*, causing the level of the fluid in *V* to rise so that fluid flows through the small tube, *t*, back into the flask, *F*.

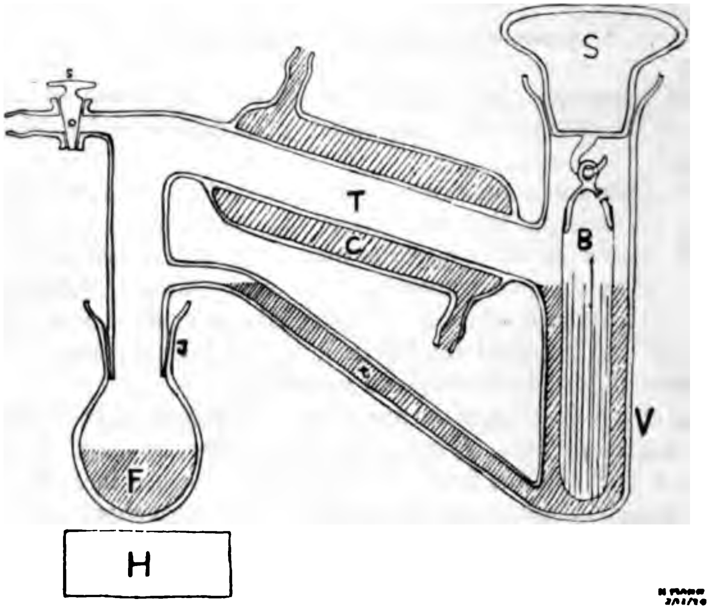


FIG. 1.

Thus the vessel, *V*, is continuously receiving a supply of freshly distilled solvent and discharging its previous content into the flask, *F*, in which flask continuous evaporation of fluid and concentration of non-volatile matter are taking place.

The advantages of the apparatus are:

1. That a small amount of solvent can be used for the extraction of a large amount of material; that this extraction can be made as complete as desired; and that the substances extracted are at the same time concentrated.

2. That this apparatus, being operated at reduced pressure, is especially suitable for the extraction of labile substances which are destroyed either by oxidation or by high temperature. With water as the solvent and the apparatus well exhausted it is not necessary to warm the flask, *F*, more than 10–15° above room temperature.

3. By using a collodion bag or other suitable dialyzing membrane it is possible to extract and concentrate the diffusible substances in plant and animal tissues; as, for example, the amino-acids of blood, muscarin, and other thermolabile alkaloids from fungi, and thus to obtain many substances of pharmacological importance.

AN ATTEMPT TOWARD THE DETERMINATION OF THE TOTAL ALKALINE RESERVE OF THE BODY.

BY W. PRENTICE, H. O. LUND, AND H. G. HARBO.

(*From the Physiological Laboratory, University of Minnesota Medical School, Minneapolis.*)

(Received for publication, August 31, 1920.)

It seems pretty well established that the alkaline reserve of human blood plasma is about 0.03 N and that of the dog is about the same (or slightly lower, especially if breathing is increased by any means when the blood is drawn). There seems to be no reliable direct method of determining the alkaline reserve of the cells of the body, but we found that on injecting acid into the blood stream the cells give up alkali to the blood plasma for the alkaline reserve, lowered by injecting acid, rose again after the acid was stopped. It seems probable that the bones should be included in the alkaline reserve of the body, but since they dissolve in scurvy when acidosis is absent this subject needs further study. The object of this study is to determine the maximum amount of acid that can be injected into the blood stream of a dog without more than neutralizing the alkaline reserve of the blood plasma. Under such conditions it was thought that the alkaline reserve of the whole body would be drawn upon.

A very large number of experiments were made, but the first and second series merely pointed out the difficulties to be met and are not given here. Pain, ether, chloroform, and the initial stage of morphine anesthesia seemed to lower markedly the alkaline reserve of the blood plasma (by inducing increased lung ventilation). Attempts at local anesthesia were abandoned. The alkaline reserve of blood drawn without anesthetic was about 0.025 N, which may be a low figure due to pain. Some dogs were kept alive several days during the experiment, but it was found that more acid could be introduced before death the more rapidly it was introduced, so that in the third series of experiments (the ones reported in this paper) the acid was introduced rapidly.

If we assume that the acid injected into the dog was equally distributed throughout the body it is important to know the ratio of the blood plasma volume to the volume of the body. Although the blood volume varies as the surface and not the volume of the body, our experiments were not sufficiently uniform to warrant accurate calculations and we merely assumed that the specific gravity was unity and the volume of the blood was 7.7 per cent of the body weight and blood plasma volume was 64 per cent of the blood volume. In other words, the volume of the body is assumed to be about twenty times the volume of the blood plasma, and if the alkaline reserve of the whole body is the same as that of the blood plasma and free diffusion takes place it would require twenty times as much acid to neutralize the alkaline reserve of the blood plasma *in vivo* as is required to do it *in vitro*. The largest amount of acid injected was 10.6 times the amount required to neutralize the alkaline reserve of the blood plasma *in vitro*, and this amount reduced the alkaline reserve *in vivo* to 0.005 N. The experiments are given in Table I.

TABLE I.

Alkaline reserve of plasma.			Acid injected.	No. of times acid would neutralize plasma.
Before ether.	After ether.	After HCl.		
			cc.	
0.023 N	0.018 N	0.0005 N	1,200	10.6
0.024 "	0.018 "	0.000 "	610	9.5
0.024 "	0.018 "	0.000 "	800	8.5
0.0235 "	0.016 "	0.000 "	740	6.9
0.023 "	0.018 "	0.000 "	700	5.8
0.024 "	0.018 "	0.000 "	400	3.63
0.025 "	0.016 "	0.000 "	750	3.33
0.0255 "	0.0175 "	0.005 "	300	3.13
0.024 "	0.0165 "	0.0025 "	300	2.9
0.0235 "	0.0175 "	0.0005 "	477	2.89

The dog was induced to lie quietly, a sample of blood was taken from a vein in his leg, and the alkaline reserve of the plasma determined. Ether was administered and a cannula inserted in the external jugular vein to allow the acid when introduced to flow in the direction of the blood stream. Another cannula was placed in the same vein at a higher level and pointed so that blood could be drawn through it, and a sample taken for alkaline reserve. The acid was 0.1 N HCl in 1 per cent NaCl and it was warmed to body temperature and passed into the vein through the first cannula from a burette at a uniform rate. When signs of coma appeared and the heart action became very weak, the flow of acid was stopped and a third sample of blood taken from the vein. We did not take intermediate samples in the first and second series of experiments because we found that the alkaline reserve could not be reduced to zero without rapid death and that there was no danger of producing a negative alkaline reserve.

The alkaline reserve was determined by the titration method of Van Slyke, Stillman, and Cullen.[1] Enough 25 per cent solution of potassium oxalate was allowed to dry in the collecting tube to make 0.2 per cent of dry oxalate in the blood. The blood was drawn rapidly and the tube closed and centrifuged. 1 cc. of plasma was introduced into a 120 cc. Erlenmeyer flask with 20 cc. of H_2O, 4 cc. of 0.01 N HCl, and 0.03 cc. of a 0.04 per cent solution of phenolsulfonephthalein. The flask was rotated rapidly by a motor for 2 minutes, during which time the CO_2 was blown off with a blast of CO_2-free air, and the contents were then titrated with 0.01 N NaOH to match the color of a similar flask at $pH = 7.2$.

---

[1] Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1919, xxxviii, 167.

VARIATION IN THE CHOLESTEROL CONTENT OF THE SERUM IN PNEUMONIA.

By HAROLD A. KIPP.*

(From the Pathological Laboratories, University of Pittsburgh, Pittsburgh.)

(Received for publication, July 20, 1920.)

The cholesterol content of the serum in acute inflammatory processes in the body has been found to undergo certain variations. A rather common agreement is found among various workers that the cholesterol content of the blood is decreased during the progress of the acute inflammatory process in the body and that it rises to normal or above normal during the period of convalescence. The fact that cholesterol possesses certain antitoxic and antihemolytic properties gives a particular interest to the variation in cholesterol in the serum in these acute infections, where the body is subjected to toxic products of invading bacteria and to the degeneration products of tissue destruction during the inflammation which ensues.

The purpose of this work was to determine the variation in the cholesterol content in the serum of patients with lobar pneumonia and to ascertain if possible the cause of this variation and the relation it bears to the inflammatory process.

Wacker and Hueck (1) report a decrease in the cholesterol content of the blood in acute infections, cavernous phthisis, and ulcerating tumors. The acute inflammatory complication of the last two is probably analogous to the first in its influence on the cholesterol content of the blood. Chauffard, Richet, and Grigaut (2) state that in tuberculosis the cholesterol content of the blood depends upon the character of the disease, whether apyretic or febrile. In the afebrile tuberculosis it remains normal, while in the febrile form the cholesterol content of the blood is constantly lowered, and markedly so when the general condition is very bad or the fever very high. The depression of the cholesterol in the blood is evidently proportionate to the intensity of the disease.

---

* R. B. Mellon Fellow in Pathology.

In following the variation in the cholesterol content of the blood in the course of typhoid fever, Chauffard, Laroche, and Grigaut (3) found that a primary hypocholesterinemia is succeeded by a gradual rise above the normal content, attaining a maximum on the 27th to the 56th day, before the patient is placed on an increased diet, and falling again to a normal figure on approximately the 73rd day. A sharp fall in the cholesterol content of the blood occurred in relapses and in perforation. This sudden lowering of the cholesterol content in acute infections, concomitant with the perforation or in reinfection with the typhoid or other organisms in the case of the relapse, is no doubt identical with the same variation in other acute infections.

In cases of obstructive icterus where as a rule the cholesterol content of the blood is increased, Rothschild and Felsen (4) find that this increase does not take place when associated with acute infections. Denis (5) found a marked diminution of cholesterol in the blood in acute infections, with a rise to normal in convalescence. However, no consecutive analyses through the course of the diseases were recorded. Isolated determinations showed from 129 to 151 mg. of cholesterol per 100 cc. of blood before crisis in pneumonia and 166 mg. per 100 cc. of blood after crisis. The cholesterol content of the blood in two cases of bronchopneumonia was recorded as 151 and 171 mg. per 100 cc. of blood, in the acute stage of the disease. In a case of pleuritis 279 mg., and in one with effusion, the character of which was not recorded, there were found 145 mg. of cholesterol per 100 cc. of blood. On account of the wide variation even in the normal cases it is evident that single analyses of blood or its component parts for the cholesterol content can give at best only a relatively accurate idea of the variation of cholesterol in the patient's blood. This emphasizes the necessity of serial analyses on the same patient in different stages of the disease.

All the foregoing results demonstrate the fact that cholesterol is diminished in that stage of disease where an acute inflammatory reaction is in progress in the body. The lowering of the cholesterol content of the serum at a time when the immune reactions of the blood are low in serum and rising again after crisis when various defense reactions are appearing in the blood and the patient is overcoming the disease, suggest a possible relation of cholesterol in the blood to the immune state and the recovery from pneumonia.

Heretofore the antitoxic property of cholesterol has been of interest and studied in connection with its ability to inhibit the hemolysis of erythrocytes by various hemolytic and toxic agents, such as oleates (6, 7), tetanolysin (8), and of importance in this investigation the pneumococcus hemotoxin (9). Chauffard and coworkers (2) suggested that the rise in the cholesterol content of the blood in typhoid convalescence is an index of its antitoxic action and parallels the acquisition of a degree of immunity in the disease. That the action of cholesterol in the recovery from disease is dependent upon its antitoxic properties has not, however, been demonstrated. Its possible rôle in the production of immunity in the body is given further light by the work of Warden (6) who found that the addition of cholesterol to fat antigens consisting of the sodium salts of specific fats of bacterial cells enhanced the value of the antigens. He also states that an antigen prepared from the cholesterol esters of fatty acids of pneumococci, injected into rabbits, gave rise to antibodies which gave greater protection to rabbits than those of sodium and lithium esters (10). This work is borne out by that of Morato and Villanueva (11), who state that the mixture of cholesterol with typhoid vaccine before injection into rabbits seemed to hasten and enhance the production both of antibodies and agglutinins and to reduce the general reaction to the antityphoid vaccine. These authors state that the administration of cholesterol in toxic infections where the blood cholesterol is low is apparently beneficial.

With this review of the apparent action of cholesterol as an adjuvant in the production of passive immunity, the question arises of the type or character of immunity developed in human blood in pneumonia. We have the work of Clough (12), who finds that after crisis or lysis in acute lobar pneumonia the serum of the patient develops definite though feeble protective power for mice, against homologous strains of pneumococci isolated from the patient whose serum was being tested. This activity was not demonstrated in the active stages of the disease in the serum, nor was it present in fatal cases. In six out of eleven cases in the convalescent stage a phagocytic activity was shown which paralleled the protective power of the serum. Phagocytosis *in vivo* paralleled phagocytosis *in vitro*. Another factor, suggested by Lord (13), may be of considerable importance in bringing about crisis and resolution of the pneumonic process. He attributes the recovery to local biochemical changes, in the course of which the acid death-point of the pneumococcus is reached.

Chickering (14) found agglutinative activity developed only at or near crisis. This agglutinative activity was limited to homologous type strains with which the patient was infected. This development of homologous agglutinins is also seen in the work of Warden (6), who found that the animal body is sensitive to minute variations in the fat complexes of certain bacterial cells and can distinguish between related members of the same species.

The important part taken by the leucocytes in the resistance of animals to experimental pneumonia has been shown by Kline and Winternitz (15). Animals treated with benzene, a leucotoxin, rapidly succumb to the disease and those treated with toluene, which causes no leucopenia, showed no decreased resistance. Nutrose induced hyperleucocytosis before infection apparently increased the resistance of the animals. In fatal cases where pneumococci had been injected intravenously in the dog, Bull (16) found that there was little phagocytosis, while in the convalescent cases the pneumococci were actively phagocyted. The leucopenia of influenza infections is in direct agreement with the above findings for in these cases pneumonia was prone to complicate the clinical course of the disease.

In a study on the experimental production of pneumonia in monkeys, Blake and Cecil (17) found that the onset of the disease is accompanied by an initial leucocytosis. This preliminary leucocytosis, which was polymorphonuclear in character, usually began within 6 hours after the injection of the pneumococci intratracheally, and reached its maximum within 24 to 48 hours later. Following the preliminary rise in leucocytes there was noted a constant fall in the curve. The rapidity and extent of this fall seemed to bear a direct relation to the severity of the disease and the degree of pneumococcus invasion of the blood.

In cases progressing to recovery, the leucocytic curve instead of continuing progressively downward turns upward again and rises in the latter half of the disease until crisis occurs, when it again falls to normal. It is worthy of note that the leucocytes are relatively few in number in the blood at the time when cholesterol is lowest and that as they increase in the latter half of the disease cholesterol is found in increased quantity in the blood serum.

With the increase in the phagocytic power of the leucocytes at the time of and following crisis, the part that these elements of the tissues and blood play in the resistance of the body to infection and recovery from the pathological state produced by the presence of parasites and their toxic agents in the body, assumes a position of interest and importance. The definite relation between the activity of the leucocyte and resistance to and recovery from the disease is shown in the foregoing work and whatever other factors may be concerned in the mechanism of recovery from pneumonia, the white blood cells play a leading rôle.

The fact that leucocytes contain a considerable amount of free cholesterol, five times as much as the erythrocytes (1), is of interest in that its presence in the leucocytes may indicate the part that cholesterol plays in phagocytosis in acute infections in the neutralization of toxic substances phagocyted by the leucocytes, so that their vitality is prolonged. That cholesterol appears to accumulate in the white blood cells during their phagocytic activities, in the course of acute inflammatory processes, follows from the analyses of Lilienfeld (18), who found 4.4 per cent of cholesterol as a component of the solids of normal leucocytes obtained from thymus. Halliburton's (19) analysis of two samples of dried pus cells by the same method gave cholesterol 7.4 and 7.28 per cent or approximately twice the content of the normal cells from thymus. Schäfer states that this large increase in the cholesterol along with the increase of lecithin and fat is in agreement with the demonstration of fatty degeneration by the microscope.

Further support of this premise suggesting the relation between the phagocytic activity of the leucocytes and the cholesterol is found in the work of Klotz (7), who states that the phagocytic activity of the vascular endothelium for fat is enhanced by the presence of cholesterol. Moreover Chauffard, Richet, and Grigaut (20) suggest that, because of its colloidal nature, the variation in cholesterol in the blood serum is related to the activity of the cells, as seen in the localized concentration of cholesterol in atheromata and xanthelasma.

With this conception of the relation between the phagocytic activity of cells and the cholesterol content of the serum, attention is drawn to the work of Christian (21), who showed that numerous polymorphonuclear leucocytes seen in the lung in the stage of gray hepatization contain a large amount of fatty material which differs in some respects from the body fat. The presence of such a large amount of fatty material in the lung at this stage of the disease allows the accumulation of a considerable amount of fatty acids which are unsaturated and toxic and are largely of the oleic acid type (22).

That the exudate of human pneumonic lungs in the stage of gray hepatization is extremely toxic for guinea pigs and rabbits, on intravenous or intramuscular injection and markedly hemolytic for guinea pig erythrocytes, has been shown by Solis-Cohen, Weiss, and Kolmer (23). These toxic substances in the lung in the stage of gray hepatization with early autolysis of the exudate are largely removed by passage through the Berkefeld filter and their hemolytic activity is neutralized by antipneumococcus serum and normal rabbit serum (24).

These authors who determined the toxicity of the lung extracts believe that the toxic principles are related to the protein fraction of the exudate, but in view of the toxicity possessed by the unsaturated fatty acids and their esters, a study of the lung in the stage of gray hepatization stained with Sudan III will impress the observer with the reasonable probability of the toxicity being in part dependent upon the presence of the large amount of foreign fat which is contained in the lung at this stage of the disease.

Typical lobar pneumonia having a rather distinct clinical picture offered a good type of acute infection for studying the varying content of blood cholesterol during the different stages of the disease. Unfortunately the bronchopneumonic type of the disease was the more prevalent and difficulties were presented in establishing a definite relation of the cholesterol content of the serum to a certain stage of the disease with a varying clinical picture. Although the number of cases presented is insufficient to establish a constant curve, certain results have been obtained which express the variation of cholesterol in the early and convalescent stages of the disease.

For the determination of the cholesterol content of blood serum in the course of pneumonia specimens of blood were obtained from patients in the wards of the Mercy and St. Francis Hospitals, through the kind. cooperation of the laboratory services of these institutions. After rimming the blood, the serum was allowed to separate and was kept in the refrigerator until the estimations were performed.

The method used in the determinations of the total cholesterol in the serum was essentially that of Bloor (25) and as later modified by him (26). On account of slight modifications which may be helpful, a brief résumé of the method is given.

The clear serum was used. The quantity varied from 1 to 3 cc. The serum, in a slow stream of drops, was delivefed from a calibrated pipette, into a 100 cc. volumetric flask which contained about 70 cc. of a mixture of one part of redistilled sulfuric ether to three parts of redistilled 95 per cent ethyl alcohol. After thorough shaking, this material was heated to boiling with constant agitation on a water bath and allowed to stand well corked for 24 hours. The quantity was then made up to 100 cc. by the further addition of the alcohol-ether mixture, mixed well, and filtered into a clean glass bottle, which contained about 5 gm. of anhydrous sodium sulfate, for the purpose of removing coloring material. The extract was mixed well with the sodium sulfate and allowed to stand well corked for 24 hours.

According to the quantity of serum used, 10 to 30 cc. of this alcohol-ether extract were delivered by a calibrated pipette into a 60 cc. glass beaker and evaporated to almost dryness on an electric hot plate. The drying was completed in a desiccator over calcium chloride. The cholesterol was estimated in triplicate samples of the alcohol-ether extract, of each specimen of serum.

The dried extracts were then taken up in about 10 cc. of chloroform, Merck's C.P. (redistilled), and boiled down to a small volume for three or four successive times. The chloroform extract was decanted each time into a calibrated measuring cylinder, and the quantity was finally made up to 5 cc. by the addition of the chloroform. The standard was prepared from a solution of Kahlbaum's cholesterol, 2 gm. in 200 cc. of chloroform. 10 cc. of this standard were added to chloroform in a 100 cc. volumetric flask and the quantity was made up by the addition of chloroform to 100 cc. at 20°C. These standard solutions were kept in the refrigerator to prevent evaporation or deterioration on exposure to light. 5 cc. of this standard

TABLE I.

Case.	Type of infection.	Onset.	Temperature.	Leucocyte counts.	Total cholesterol per 100 cc. serum.	Progress.
			°F.		mg	
No. 1. Age 44 yrs. Admitted Jan. 10, 1920.	Type IV. Pneumococcus pneumonia.	Jan. 10. Cough, rusty sputum, pleuritis, fever.	2.* 103 3. 103 4. 104 5. 102 6. 102 8. 102 9. Normal.	3.* 19,000 4. 10,500 5. 14,000	8.* 123 10. 200	2.* Consolidation right lower lobe. 4. Consolidation right lower lobe. 6. Coarse râles in area of consolidation. Recovery.
No. 2. Age 23 yrs. Weight 160 lbs. Admitted Dec. 3, 1919.	Type IV. Pneumococcus lobar pneumonia.	Dec. 1. Chill, fever, cough, pleuritis.	3. 101 4. 103 5. 104 Lysis. 8. 101 Lysis. 18. Normal.	4. 4,000 5. 8,500 6. 10,000 7. 14,000 8. 13,000 12. 26,900 13. 15,600	8. 155 18. 265	5. Dyspnea, delirium, very toxic; consolidation right upper lobe, patchy consolidation left lower lobe. 13. Coarse râles in area of consolidation; general condition much improved. 17. Improvement continued. Resolution. Recovery.

* Day of disease.

Admitted Jan. 26, 1920.	mococcus *B. influenza* lobar pneumonia.		8. 102–97 9. Normal.	8. 8,000	9. 215 20. 257	9. Left lower lobe consolidated. 18. Chest clear for several days. 19. Allowed out of bed. Recovery.
No. 4. Admitted Feb. 3, 1920.	Type IV. Pneumococcus *B. influenza* *Streptococcus viridans* bronchopneumonia.	Feb. 1. Headache, pleuritis.	3. 104 8. 101 9. 100 10. 99 11. Normal.	7. 5,600 8. 10,400 9. 17,600 10. 9,700 12. 14,600	12. 227 16. 166	9. Definite consolidation apex right lower lobe. 18. Patient feels well, allowed out of bed. Recovery.
No. 5. Admitted Feb. 7, 1920.	*Streptococcus viridans* bronchopneumonia.	Feb. 1. Chill, cough, fever, pain in joints, bloody sputum.	6. 100 7. 104 8. 98 9. Normal.	9. 6,120	11. 152 18. 227	6. Increased conduction right chest. 8. General condition improved. 9. Lungs clear. 14. Patient out of bed. Recovery.

TABLE I—*Continued.*

Case.	Type of infection.	Onset.	Temperature.	Leucocyte counts.	Total cholesterol per 100cc. serum.	Progress.
			°*F.*		*mg.*	
No. 6. Admitted Dec. 29, 1920.	Type II. Pneumococcus lobar pneumonia.	Dec. 27. Chill, fever, pleuritis.	3. 104	3. 11,600		3. Cyanosis, dyspnea. Both bases early consolidation.
			4. 103	4. 6,600		
			Lysis.	7. 8,000		7. Spleen enlarged, left lower lobe shows more consolidation than the right lower.
			12. Normal.	12. 15,000	14. 231	
						9. Beginning resolution left base.
					24. 304	18. Patient much improved. Recovery.
No. 7. Admitted Jan. 9, 1920.	Type IV. Pneumococcus *B. influenza* central pneumonia.	Jan. 6. Fever, cough, pleuritis.	3. 102	3. 28,000		3. Friction rub right lower chest.
			5. 102			7. Slight dullness right chest at angle of scapula.
			6. 100	6. 13,400		
			8. 100	7. 16,800		
			9. 99	8. 12,000		8. Clear fluid (5 cc.) aspirated from right pleural sac. Gram-positive diplococci present.
			10. Normal.	9. 10,000	9. 159	
				15. 9,600	13. 221	
					18. 245	9. Scattered moist râles over left lower lobe.
					26. 333	
					23. 200	13. Chest clear.

			15.	99			19	217	12. Resolution continuing.
			22.	99					23. Chest clear.
			25.	99	26.	8,100			
			28.	Normal.			34.	221	Recovery.
No. 9. Admitted Mar. 21, 1920.	Bronchopneumonia.	Mar. 19. Headache, cough, pleuritis, bloody sputum.	3.	104					3. Cyanosis, dyspnea. Beginning consolidation of right lower lobe, and right upper lobe.
			4.	102			4.	153	
			6.	101	5.	16,800			
			8.	101					7. Right upper lobe consolidated.
			9.	100					9. Beginning resolution.
			10.	101					17. Resolution in right lower lobe.
			11.	Normal.					19. Right lower lobe clear.
			16.	100					25. Chest clear. Allowed up.
			17.	Normal.	19.	19,200			
							26.	168	
							33.	221	Recovery.

TABLE I—*Concluded.*

Case.	Type of infection.	Onset.	Temperature.	Leucocyte counts.	Total cholesterol per 100cc. serum.	Progress.
			°*F.*		*mg.*	
No. 10. Admitted Jan. 14, 1920. Died Jan. 27, 1920.	Type IV. Pneumococcus pneumonia.	Jan. 10. Cough, pleuritis, malaise.	5. 104 6. 104 7. 103 8. 103 9. 101 10. 100 12. 103 13. 102 14. 105 16. 105 18. Death.	6. 13,000 8. 24,400 15. 20,920 18. 23,000	7. 135 11. 158 15. 193	5. Consolidation of right lower lobe. Fluid in left chest. Large moist râles at left base. 10. Dullness persists at right base. Bubbling râles throughout left and right chest anterior and posterior. Signs of circulatory failure. 16. Bubbling râles throughout entire chest. Heart sounds weak, pulse irregular. Death on 18th day.
No. 11. Admitted Feb. 10, 1920. Discharged Mar. 4, 1920.	Lobar pneumonia. Acute parotitis.	Mar. 7. Chills, repeated on 8th and 9th, pleuritis, cough.	4. 102 5. 101 7. 101 9. 100 10. 99 p.m. temperature of 1° from 12th	9. 18,800	3. 188 10. 187 16. 208 36. 201	3. Consolidation of right lower lobe. 5. Consolidation fully developed. 12. Crepitant râles at right base, resolution progressing. Parotid

9, 1920.		tis.	7. 104 8. 104–100 9. 100–98 10. Normal.		12. 152 18. 189	lower lobe. 7. Small area of consolidation at angle of left scapula. 9. General condition improving. 11. Resolution progressing in left lower lobe. 16. Lungs clear. Recovery.
No. 13. Admitted Feb. 27, 1920.	Influenza bronchopneumonia.	Feb. 24. Chill, headache, hemoptysis, pleuritis.	4. 104 9. 103 11. 100 13. Normal.	9. 15,000 10. 10,400 11. 16,120	13. 157 17. 131	4. Consolidation right middle lobe. 7. Consolidation right middle lobe. 14. Consolidation right middle lobe. 16. Resolution in progress in right middle lobe. 20. Lungs clear.

TABLE II

Case.	Type of infection.	Onset.	Temperature.	Leucocyte counts.	Total cholesterol per 100 cc. serum.	Progress.
1920			°*F.*		*mg.*	
No. 1A. Admitted Jan. 22.	Type IV. Pneumococcus bronchopneumonia.	Jan. 19. Chill, cough, pleuritis, headache.	4.* 104 6. 101 10. Normal.	8.* 2,400 10. 4,680	16.* 240 25. 240	4.* Chest full of moist râles. No areas of consolidation noted. Recovery.
No. 2A. Admitted Jan. 23.	Type IV. Pneumococcus resolved pneumonia.	Jan. 8. Crisis about 1 week before admission.	19. Normal.	19. 9,000	19. 242	18. Chest clear. Evidence of old pneumonia on right side of chest.
No. 3A. Admitted Feb. 13.	Influenza bronchopneumonia.	Feb. 10. Severe chill, fever, pleuritis, cough.	3. 102 6. 98 8. 102 9. Normal.		6. 128	3. Early consolidation of right lower lobe. 6. Consolidation of nearly entire right lung. 10. Beginning resolution of right lung. 13. Resolution progressing. 17. Chest clear. Recovery.

* Day of disease.

No. 4A. Admitted Feb. 10.	Influenza pneumonia.	Sick about 3 weeks before admission. In bed for 3 days before admission.	20. 104	20. 7,606		20. Consolidation of middle lobe of right lung.
			21. 103	21. 8,600		21. Consolidation increasing. Right lung filled with râles.
			22. 101	22. 5,100	22. 166	
			23. 98			
			24. 104			
			25. Death.			25. Death.
No. 5A. Admitted Jan. 30.	Type III. Pneumococcus bronchopneumonia.	Jan. 23. Chill, headache, pleuritis.	8. 104	8. 5,800		8. Consolidation of right lower lobe.
			9. 103		9. 142	9. Consolidation of right lower lobe. Some fine moist râles in this area.
			10. 103			
			11. 102			11. Increasing involvement of left lower and right upper lobes.
			12. 103			
			13. 103			
						Death.
No. 6A. Admitted Jan. 30.	Type III. Pneumococcus.	Jan. 26. Dry cough, pleuritis.	5. 101			5. Moist râles at right base.
			6. 100		6. 188	10. Moist râles in right lung, no consolidation.
			9. 100			
			11. 102			16. Chest clear.
			12. 103			Recovery.
			13. 99			
			15. Normal		15. 185	

TABLE II—*Concluded.*

Case.	Type of infection.	Onset.	Temperature.	Leucocyte counts.	Total cholesterol per 100 cc. serum.	Progress.
1920			°*F.*		*mg.*	
No. 7A. Admitted Feb. 22.	Type IV. Influenza pneumococcus bronchopneumonia.	Feb. 18. Headache, pleuritis, cough.	5. 105	5. 9,000		5. Consolidation of right lower lobe.
			6. 103			6. Consolidation of right lower lobe.
			7. 102			
			8. 101			
			10. 101			
			11. Normal.		17. 320	19. Lungs clear.
No. 8A. Admitted Feb. 2.	Acute bronchitis; bronchopneumonia.	Jan. 31. Cough, headache.	3. 104			3. No areas of consolidation.
			4. 102			4. Possibly a small area of consolidation at right base. Moist râles at right base.
			5. 99			
			6. Normal.		13. 242	
						15. Patient out of bed.
						Recovery.
No. 9A. Admitted Jan. 27.	Influenza bronchopneumonia.	Jan. 21. Chill, fever, cough, prostration.	7. 104			
			8. 102	8. 14,000		8. Patchy consolidation of right lung; moist râles both lungs.
			9. 102		9. 87	9. Coarse râles left upper lobe; signs of consolidation left lower lobe.
			Continued high temperature.			11. Chest signs accentuated; toxic.
						12. Pulse irregular; delirium.
						Death.

ase. 1920	Type of infection.		°F.		100 cc. serum. mg.	
Case A. Admitted Mar. 19.	Type IV. Pneumococcus *Streptococcus viridans*, *Staphylococcus albus* pneumonia. Hemolytic streptococcus empyema.	Mar. 17. Cough, pleuritis.	3.* 105 4. 103 7. 102 10. 101 11. 101 Temperature 99–102° from 11th to 38th. 38. Normal.	23.* 25,500	6.* 176 35. 129 44. 245	3.* Signs of fluid in left pleural sac. Left base consolidated. 15. 600 cc. pus aspirated from left pleural sac. 15th to 33rd day, 1,400 cc. pus aspirated from left pleural cavity. 52. Patient out of bed. Recovery.
Case B. Admitted Jan. 21.	Type I. Pneumococcus bronchopneumonia. Empyema.	Jan. 11. Chill, cough, fever, pleuritis.	11. 103 13. 104 15. 103 16. 102 17. 100 18. 100 19. 102 20. 99 21. Normal.	12. 14,000	14. 135 17. 140 21. 122 30. 308	11. Signs of consolidation of left lower lobe. Fine and coarse moist râles over left side posteriorly. 12. Distinct signs of fluid at left base. 38. Patient allowed out of bed. Recovery.
Case C. Admitted Jan. 22.	Type II. Pneumococcus pneumonia. Empyema.	Jan. 18. Cough, headache, rusty sputum, pleuritis.	5. 104 8. 104 10. 103 11. 103 12. 103 13. 103 14. Death.	6. 7,290 10. 6,560	7. 166 11. 83	5. Signs of consolidation of right middle lobe. Signs of fluid in left chest at base. 12. 400 cc. pus aspirated from left chest. Death.

---

* Day of disease

TABLE IV.

Case No.	Diagnosis.	Total cholesterol per 100 cc.			Description of material.
		A*	B	C	
		mg.	*mg.*	*mg.*	
1	Pleuritis.	103	102	1	Canary-yellow fluid. Faint cloud of suspended cells.
2	"	98	92	6	" " containing a fine web of fibrin clot in which are suspended a very few cells.
3	Empyema, postpneumonic.	203	59	154	Creamy yellow pus, containing many cells. Many Gram-positive diplococci seen in direct smear.
4	" "	304			Thick greenish yellow pus, containing many cells. Many Gram-positive cocci in chains in direct smear.
5	" "	217			Thick yellow pus. *Streptococcus pyogenes vulgaris.*
6	" "	219	36	183	Thick pus. *Streptococcus hæmolyticus.*
7	" "	362			Thick greenish yellow pus. *Streptococcus hæmolyticus.*
8	" "	342	108	234	Thick pus containing many cells.
9	" vesicæ felleæ.	231	41	190	Thick greenish pus of foul odor. *B. proteus vulgaris.*

* A = Entire exudate.
B = Supernatant fluid after centrifugalization.
C = Difference, or the cellular portion.

were added to a stoppered measuring cylinder for each estimation. After the addition of 2 cc. of Merck's acetic anhydride (colorless) and 0.1 cc. of concentrated sulfuric acid, the cylinders were placed in a water bath at a constant temperature of 22°C., for 15 minutes. The contents of the cylinders were then placed in the cups of a Duboscq colorimeter and the unknown was compared with the standard at 15. The comparison could in a majority of instances be read at this place. The presence of a brownish coloration was noted, but it did not seriously interfere with the readings. The amount of cholesterol was then computed in milligrams per 100 cc. of serum.

In Table I are given results in which two or more determinations on different days of the disease were obtained of the cholesterol content of the serum. The clinical notes accompanying the analyses were obtained from the patients' chart as recorded by the physicians in attendance on the case.

Table II gives a series of cases in which only one specimen of serum was available for analysis, with the exception of two cases which showed no variation in the cholesterol content of the serum.

In Table III are given three cases of pneumonia which developed empyema.

In Table IV are the results of analyses of a number of pleural exudates and of one empyema of gall bladder. These exudates varied in character. The analyses showed the difference in the cholesterol content of the fluids and the cellular portion of the exudate which is probably similar to that of the exudates in the lung in the stage of gray hepatization, and presents a more readily available form of material upon which estimations are made in the same manner as with the blood serum.

DISCUSSION.

Cases 1, 2, 3, 5, and 6, in which the patients developed a well defined pneumonic consolidation, showed a subnormal cholesterol content in the serum in the acute stage of the disease and a much higher cholesterol content during convalescence when resolution in the lung had been in progress for some time. Case 7 showed a variation in the cholesterol content of the serum from a low figure before resolution to a hypercholesterinemia in the course of convalescence, which was later succeeded by a return to a normal content. The variation in this case resembles very much the contour of the curve which Chauffard (3) established in typhoid fever. Case 10, with a continued high temperature and with resolution progressing in the lung, showed a rise in the cholesterol content of the serum paralleling the process of resolution of the pneumonic exudate. Cases 4 and 13 demonstrate a return to normal in the cholesterol content of the serum subsequent to a hypercholesterinemia. The difference in time of the return of the cholesterol content of the serum to normal in these two cases and in Case 7 indicates that there is a marked difference in the cholesterol variation in each individual case, dependent upon the intensity of the disease and the extent of the inflammatory reaction.

Although each case showed a primary hypocholesterinemia with a rise in the cholesterol content of the serum in convalescence, a hypercholesterinemia in convalescence is demonstrated only in Cases 4, 7, and 13. The tendency of the variation of the cholesterol content of the serum in the other cases is also toward a hypercholesterinemia in convalescence and probably this variation obtains in the majority of cases where there has been a considerable degree of consolidation of the lung.

The list of cases in which single analyses were available substantiates the general findings in the cases in Table I. In three fatal cases, the cholesterol content of the serum was very low, and in the case with the greatest amount of lung involvement, the depression was lowest. This finding suggests that the depression of the cholesterol content of the serum is dependent upon the amount of involvement of the lung and may also indicate an increased utilization of cholesterol in the more severe cases. In Case 3A, which recovered, there was a marked lowering of the cholesterol content of the serum during the acute stage of the disease with a large area of lung tissue involved. In Case 7A, with a definite consolidation of one lobe, the temperature coming to normal on the 11th day of the disease, a hypercholesterinemia was present on the 17th day.

In the two cases noted, one during the acute stage of the disease and the other in convalescence in which there was no consolidation, there was no change in the cholesterol content of the serum. These figures again support the probability that the cholesterol content of the serum in pneumonia is dependent upon and parallels the activity of the white blood cells in their participation in the formation of the exudate at the site of the acute inflammatory process.

Blake and Cecil (17) have determined the curve of variation of the leucocytes in the blood in the course of experimental pneumonia. In superimposing the results of our determinations of the cholesterol content of the blood upon this curve, it is remarkable how closely they coincide. The initial leucocytosis of short duration with a subsequent fall parallels the lowering of the cholesterol content of the serum. The leucocytes at this time are gathering at the site of the acute inflammatory process.

The lung exudate in the stage of gray hepatization has been shown to be toxic for animals. The presence of the large amount of fatty substances in this exudate suggests this abnormal fat as responsible in part for the toxicity. The cholesterol existing in the serum as a readily available neutralizing substance in a colloid state is removed from the serum by the phagocytic leucocytes, and participates in the inflammatory process as a component of the white blood cells. At the site of the acute inflammation it performs the function of neutralizing the toxins of the organisms and the toxic products of the disease process. The subsequent rise in cholesterol in the serum is seen when the leucocytes are returning to the general circulation.

In the three cases of empyema presented, it is seen that the development of this complication in the course of the pneumonic process delays the return of the cholesterol in the serum to a normal figure. In addition, the cholesterol content of the serum is lower, on an average, than the depression of this substance in the serum in the uncomplicated pneumonia. In the fatal cases the marked lowering of the cholesterol again suggests an increased utilization of cholesterol with the extension of the inflammatory process.

Cholesterol is a substance which is strictly conserved by the animal organism. Dorée and Gardner (27) state that part of the cholesterol in bile is derived from the breaking down of the blood and tissue cells which contain a relatively large amount of it. Analogy may be drawn to this in the explanation of the variation of the cholesterol in the blood stream in the consolidation of the lung, where there is lysis of the cells of the exudate at the time of resolution, and in the formation of purulent exudates in empyema.

Analysis of the empyema fluids shown in Table IV demonstrates that the exudates containing a large number of pus cells possess from two to three times the amount of cholesterol found in the relatively acellular fluids. The exudates which contained few cells contained considerably less cholesterol than is found in normal serum showing that the presence of cholesterol in the exudates is related to the cellular constituents. In these richly cellular exudates, moreover, it was found that the fluid portion of the exudate contained even less cholesterol than the acellular fluid, indicating an accumulation of cholesterol by the cells of the fluid. Lilienfeld's (18) analyses show the accumulation of cholesterol in these pus cells. The enrichment of the cells of the exudate in cholesterol probably indicates on the other hand that these cells adsorb a considerable amount of cholesterol from the serum before migrating to the site of the acute inflammation.

CONCLUSIONS.

1. The cholesterol content of the blood serum in pneumonia exhibits the following variations:

(*a*) A primary hypocholesterinemia, the degree of which is dependent upon the degree of lung involvement and the intensity of the infection.

(*b*) A secondary hypercholesterinemia in the period of convalescence, during and continuing for a varying period of time after the resolution of the pneumonic exudate.

(*c*) A return to the normal cholesterol content of the serum.

2. The variation in the cholesterol in the blood serum in pneumonia is dependent upon the activity of the leucocytes and being transported by them to the area of acute inflammation acts as an antitoxic substance, neutralizing the bacterial toxins and those arising from the disintegration of tissue in the process of the inflammatory reaction. It also may serve as an adjuvant in the development of antigens which initiate the process of resolution of the pneumonic exudate.

3. The utilization of cholesterol in acute toxic infections is directly proportional to the severity of the disease.

4. The development of empyema definitely alters the variation of cholesterol in the serum in pneumonia.

It is a pleasure to express my sincere thanks to Dr. Oskar Klotz for suggesting the subject of this research and for his supervision throughout the course of the work.

BIBLIOGRAPHY.

1. Wacker, L., and Hueck W., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxiv, 416.
2. Chauffard, A., Richet, C., and Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxx, 276.
3. Chauffard, A., Laroche, G., and Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxx, 70.
4. Rothschild, M. A., and Felsen, J., *Arch. Int. Med.*, 1919, xxiv, 520.
5. Denis, W., *J. Biol. Chem.*, 1917, xxix, 93.
6. Warden, C. C., *J. Infect. Dis.*, 1918, xxiii, 504.
7. Klotz, O., *J. Med. Research*, 1915–16, xxxiii, 157.
8. Noguchi, H., *Univ. Pennsylvania Med. Bull.*, 1903, xv, 327.
9. Cole, R., *J. Exp. Med.*, 1914, xx, 346.
10. Warden, C. C., *J. Infect. Dis.*, 1919, xxiv, 285.
11. Morato, E., and Villanueva, F., *Med. Iberia. Madrid*, 1919, ix, 107.
12. Clough, P. W., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 340.
13. Lord, F. T., *J. Exp. Med.*, 1919, xxx, 379.
14. Chickering, H. T., *J. Exp. Med.*, 1914, xx, 599.
15. Kline, B. S., and Winternitz, M. C., *J. Exp. Med.*, 1913, xviii, 50.
16. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.
17. Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.
18. Lilienfeld, L., *Z. physiol. Chem.*, 1894, xviii, 473.
19. Halliburton, W. D., in Schäfer, E. A., Text-book of physiology, Edinburgh and London, 1898, i, 82.
20. Chauffard, A., Richet, C., and Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxx, 317.
21. Christian, H. A., *J. Med. Research*, 1903–04, x, 109.
22. MacPhedran, F., *J. Exp. Med.*, 1913, xviii, 527.
23. Solis-Cohen, S., Weiss, C., and Kolmer, J. A., *J. Infect. Dis.*, 1918, xxii, 476.
24. Weiss, C., Kolmer, J. A., and Steinfeld, E., *J. Infect. Dis.*, 1918, xxii, 469.
25. Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.
26. Bloor, W. R., *J. Biol. Chem.*, 1917, xxix, 437.
27. Dorée, C., and Gardner, J. A., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 109.

THE NATURE OF CHLORINE COMBINATION IN URINE.

BY A. T. CAMERON AND M. S. HOLLENBERG.

(*From the Department of Biochemistry, University of Manitoba, Winnipeg, Canada.*)

(Received for publication, September 13, 1920.)

Neuberg[1] states that Berlioz and Lépinois,[2] and other French writers found chlorine present in urine partly in organic combination. Later investigators[3,4] denied this. Baumgarten[5] obtained results indicating that 0.04 to 0.2 gm. of organic chlorine is excreted daily in the urine; the maximum found amounted to about 10 per cent of the total chlorine excretion, and was independent of the amount of sodium chloride in the diet and the temperature of the individual. We have been unable to find any more recent work dealing with the possible presence of organic chlorine in urine.

Baumgarten's method consisted in treating urine with sodium nitrite and fuming nitric acid (to reduce chlorates), adding excess of silver nitrate and filtering in darkness, and, after removal of excess silver, concentrating to one-third, again filtering, and heating the filtrate in a retort with concentrated nitric and sulfuric acids free from chloride. The distillate was collected in a dilute silver nitrate solution, and silver chloride was precipitated in this corresponding to the amounts of chlorine indicated above. This chlorine was considered to be in organic combination.

We have repeated Baumgarten's procedure with completely negative results.

---

[1] Neuberg, C., Der Harn sowie die übrigen Ausscheidungen und Körperflüssigkeiten von Mensch und Tier, Berlin, 1911, i, 112.

[2] Berlioz, A., and Lépinois, E., *J. pharm. et chim.*, 1894, xxix, 288.

[3] Ville, J., and Moitessier, J., *Compt. rend. Soc. biol.*, 1901, liii, 673.

[4] Meillière, G., *Compt. rend. Soc. biol.*, 1901, liii, 1174.

[5] Baumgarten, O., *Z. exp. Path. u. Therap.*, 1908–09, v, 540.

Chlorates and perchlorates do not appear to occur normally in urine, and when administered are excreted in the urine unchanged and almost quantitatively within 48 hours.[6] We have confirmed the absence of chlorine oxy-acids from normal urine by the following procedure.

The halide content of 20 cc. of urine was precipitated as silver chloride in the presence of nitric acid, the precipitate was collected on a Gooch crucible, washed with distilled water, and dried at 100°C, to constant weight. Another 20 cc. of urine was treated with nitrite and fuming nitric acid, allowed to stand for some hours, and then the halide content estimated as just described. The results indicate that chlorine oxy-acids can be considered as absent from normal urine (Table I).

TABLE I.

Subject No.	Chloride-chlorine in 100 cc. urine.	Chlorine and oxy-acid chlorine in 100 cc. urine.
	gm.	*gm.*
1	0.7832	0.7837
	0.7837	0.7843
2	0.6226	0.6233
	0.6220	0.6225
3	0.5646	0.5649
	0.5650	0.5653

We have made a careful comparison of the chloride (halide) content and the total chlorine (halogen) content of urine. The halide in 20 cc. of urine was estimated as usual. The total halogen was estimated by evaporating 20 cc. of urine with 10 gm. of solid sodium hydroxide, heating the fused mass with additions of very small quantities of potassium nitrate until all organic matter was oxidized, dissolving in water, and estimating the halide as silver chloride as usual. The results are given in Table II.

The slightly greater differences with the pathological urines were probably due to difficultly filterable organized matter, retained in the Gooch crucible but destroyed during fusion. The close agreement indicates the absence of organic chlorine.

[6] Heffter, A., *Ergebn. Physiol., 1te Abt.*, 1903, ii, 108.

TABLE II.

Subject No.	Condition.	Chloride-chlorine in 100 cc. urine.	Total chlorine in 100 cc. urine.
		gm.	*gm.*
1*	Normal.	1.0835	1.0834
		1.0836	1.0831
		1.0831	1.0834
2	"	0.6227	0.6235
		0.6216	0.6232
4	"	0.9383	0.9365
		0.9358	0.9387
5	"	0.9451	0.9449
		0.9447	0.9444
6	Tubercular.	0.3034	0.3028
		0.3041	0.3037
7	"	0.4876	0.4867
		0.4880	0.4864
8	"	0.4480	0.4474
		0:4477	0.4476
9	"	0.3670	0.3637

* A fresh sample.

All our figures are for halogens and halides (estimated as chloride). As under normal conditions only traces of bromides and iodides are present in urine, the results can be considered in terms of chlorine and chloride.

We conclude that in the absence of chlorate or similar medication chlorine occurs in urine as chloride only; organic chlorine and chlorine oxy-acids are absent.

THE FAT CONTENT OF EMBRYONIC LIVERS.

By C. G. IMRIE and S. G. GRAHAM.

(*From the Department of Pathological Chemistry, University of Toronto, Toronto.*)

(Received for publication, August 23, 1920.)

Fatty infiltration of the liver may be produced experimentally in animals by the injection of such substances as phlorhizin, myristicin, and oil of pulegone; also by fasting. The fat which accumulates in the liver under such conditions is believed to be derived from the subcutaneous tissues and other depots where fat is stored, for it has been shown that as the fat accumulates in the liver it resembles more and more in its degree of desaturation the fat of the connective tissue. This may be readily shown by a determination of the iodine value of the higher fatty acids extracted from the particular tissue. The histologically invisible fat which is normally present in the liver and other organs of animals and which enters into the physical structure of the cell, is much less saturated and has therefore a higher iodine value than the fat deposited in comparatively inert form in the connective tissue. As the amount of fat in the liver increases, the iodine value is lowered until in livers where large amounts of fat are present the iodine value of the fat very closely approximates that of the connective tissue fat.

While engaged in the study of the changes thus effected in the organs of animals, it seemed desirable to extend the observations to pregnant animals and to determine the relation in which the embryonic liver stood to mobilized fat. It seemed reasonable to suppose that the embryonic tissue might possess an avidity for food material such as fat and that this might evidence itself by a greater accumulation of fat in the embryonic than in the maternal liver, if the fat were mobilized experimentally.

EXPERIMENTAL.

Because of their convenient size and prolific tendencies, guinea pigs were selected for the observations. The higher fatty acids were determined by the method of Leathes (1) and the iodine value by the method of Wys (2).

A few preliminary determinations showed that the percentage of fat in the embryonic liver was not constant, but varied with the age of the embryo. The livers obtained from young embryos, weighing about 10 gm., histologically and chemically resembled the adult hepatic tissue, in so far as their fat content was concerned, whereas in embryos fully developed the liver was yellow in color and chemically showed a large accumulation of fat. These findings suggested a physiological infiltration of fat in the embryonic liver during development, and obviously, if such were the case, it was necessary to establish its nature before correct interpretations could be made of observations under experimental conditions.

In order to ascertain the nature of this fat accumulation, pregnant animals in various stages of gestation under normal conditions were killed, and the liver tissue was examined as previously described. Where the embryos were very small, the livers from the members of one litter were pooled and one determination was made upon the lot in order to obtain, if possible, sufficient fatty acid for an accurate iodine value determination.

In Table I the results from twenty-two guinea pigs are recorded. They are arranged in the order of the increasing weights of the embryos. In nineteen instances, the values obtained from the maternal livers are also given. The weight of the embryo has been employed as an indication of its age, and this, while for obvious reasons somewhat unsatisfactory, has proved sufficiently accurate for our purpose.

These figures show quite definitely that a fatty infiltration takes place in the livers of embryo guinea pigs. Until the embryo reaches an age corresponding to a weight of about 40 gm., there is little or no evidence of this, but as its weight increases beyond this the increase in fat is very marked. The average fat value of the liver in embryos under 40 gm. in weight is 3.16 per cent, whereas in embryos over 40 gm. in weight it is 13.10 per cent.

It seems that the rate of infiltration is most marked during that period of development represented by weights of 40 to 60 gm.

In Table II, the figures have been obtained in the following way. The embryos have been divided into nine groups according to their weights. Group 1 includes those under 10 gm.; Group 2 includes those over 10 gm. and under 20; Group 3 those over 20 gm. and under 30; and so on, while Group 9 includes those over 90 gm. In each group, the average weight of the embryos, the average fat content, and iodine value of the embryonic and maternal livers have been calculated and expressed. The infiltration of fat into the embryonic livers is well demonstrated in these averages.

TABLE I.

Weight of embryos.	Embryonic livers.		Maternal livers.	
	Higher fatty acids.	Iodine value.	Higher fatty acids.	Iodine value.
gm.	*per cent*		*per cent*	
3.0	2.39		1.86	117
5.4	3.49		3.52	94
6.0	3.40	118	1.44	117
13.0	3.35	114	2.03	122
13.0	3.08	102	2.50	111
23.5	2.84	98	2.31	123
24.0	2.40	106	2.34	120
29.0	3.63	105	2.15	116
32.0	4.12	113	2.25	114
37.2	2.88	115	2.24	120
42.0	5.06	107	2.14	126
50.0	9.60	110	1.17	115
55.5	14.29	112	1.69	123
64.0	12.27	109	1.69	123
68.0	15.80		2.66	120
70.0	12.00	103		
72.0	11.46	101	1.81	112
72.0	14.60	92	3.54	117
73.0	15.70			
90.0	16.15	105	3.03	94
101.0	15.14	100	2.41	118
117.0	15.22	102		

It is of special interest to note that this infiltration is not associated with any such change in the maternal liver. The highest fat value in the maternal livers in the series was 3.54 per cent, and in only three instances were the values over 3 per cent. The average fat content of the maternal livers in animals with embryos under 40 gm. in weight was 2.26 per cent, while that obtained in animals with embryos over 40 gm. in weight was 2.24 per cent. Thus, the average amount of fat in the embryonic liver may increase from 3.16 per cent in the first period to 13.10 per cent in the second period, as previously mentioned, while in the maternal liver the average amount of fat remains practically unaltered. This seems to indicate that the infiltration of fat in the embryonic liver is an embryonic process and that this organ possesses an avidity for fat not possessed by the maternal liver at that time.

TABLE II.

Group No.	Average weight of embryos.	Embryonic livers.		Maternal livers.	
		Average higher fatty acids.	Average iodine value.	Average higher fatty acids.	Average iodine value.
	gm.	*per cent*		*per cent*	
1	4.8	3.09		2.27	109
2	13.0	3.22	108	2.27	116
3	25.5	2.95	103	2.26	119
4	34.62	3.50	114	2.24	117
5	42.00	5.06	107	2.14	126
6	52.50	11.95	111	1.42	119
7	67.30	13.35	106	2.05	118
8	72.30	13.92	96	2.67	114
9	102.70	15.50	102	2.72	106

A consideration of the iodine value affords a point of interest. The average iodine value of the fat in the livers of embryos under 40 gm. in weight is 109. Some of the figures from which this average has been calculated are probably a little low because in those particular instances the livers were too small to provide an adequate amount of fatty acid for an accurate iodine value determination. However, even allowing for this, the evidence in favor of the point which we wish to bring out is sufficiently convincing. The average iodine value of the fat in the livers of embryos over 40 gm. in weight is 104. In brief, therefore, while in embryos under 40 gm. in weight the average higher fatty acid content of the liver amounts to 3.16 per cent having an iodine value of 109, in embryos over 40 gm. in weight the average higher fatty acid content of the liver is 13.10 per cent having an iodine value of 104. This accumulation of fat in the embryonic liver is not accompanied by any marked decrease in the iodine value or approach to the value found in the connective tissue fat of the guinea pig which is 85.

As previously mentioned, it is known that, in the human liver and in that of other animals such as the dog and cat, an infiltration of this organ with fat is accompanied by a lowering of the iodine value of the fat. As has been shown by Imrie (3), the actual values in human cases approach very nearly to those which may be calculated, if it is assumed that the fat found over and above the average value had an iodine value similar to that of the connective tissue fat.

The difference between the above average fat values of 3.16 and 13.10 per cent is 9.94 per cent. If we assume that this was fat infiltrated from the connective tissue and having an iodine value of 85, and added to the 3.16 per cent having the mean iodine value found, namely 109, the resulting iodine value should be 91; but instead it is 104. In only one instance does the value actually obtained coincide with the theoretical value and in this particular instance there is evidence of some fatty infiltration in the maternal liver, the influence of which we shall discuss later. This seems to show, therefore, that the fat is less saturated than would be the case if the access of fat were due merely to an influx of connective tissue fat.

Leathes and Meyer-Wedell (4) believe that it is a function of the liver to desaturate fat brought to it, to introduce double linkages into the molecule, and thus prepare it for subsequent oxidation. When a fatty infiltration occurs owing to an abnormal influx of connective tissue fat, the liver falls into arrears in its work, the fatty acids are no longer desaturated to the normal extent, and a low iodine value is accordingly found.

The results here given, however, seem to indicate that at a certain stage in the development of the embryo there is a very marked increase of fat in the liver and this fat has not the character of connective tissue fat. The explanation of this difference may be either that the embryonic liver can desaturate larger amounts of imported fat than the liver in extrauterine life or that the fat taken up by the liver of the embryo is fat which has already been desaturated by the maternal liver or other tissues.

Having observed this physiological infiltration of fat, the next point we considered was the ultimate fate of this fat. If the purpose of this accumulation of fat was to provide a readily available source of food for the embryo, to be utilized immediately after birth, it seemed reasonable to suppose that the fat would decrease rapidly during the first 2 or 3 days of extrauterine existence.

The fat content of the livers of embryos, members of the same litter, is comparatively uniform. In Table III are recorded the results obtained from the examination of four litters.

TABLE III.

Litter No.	Weights of embryos.	Higher fatty acids in livers.	Iodine value.
	gm.	*per cent*	
1	55.5	14.29	110
	64.0	12.47	109
2	70.0	12.00	103
	72.0	11.46	101
3	72.0	14.6	92
	73.0	15.7	
4	101.0	15.14	100
	117.0	15.22	102

Litters of guinea pigs after birth were examined in the following manner. One guinea pig was killed about 16 hours after birth, the second guinea pig of the same litter was killed 24 hours after the first, and the third guinea pig, when such existed, was killed 24 hours after the second. In each case the fat content of the liver and its iodine value were determined. In all, eight litters were examined. Four of these were composed of three guinea pigs and four of two guinea pigs. The results are recorded in Table IV.

In each litter there is a marked decrease in the fat values during the first 2 or 3 days of life. Litters 1, 2, and 8 show this very strikingly. The decrease found in Litter 8 is most marked. An exceptionally high value for the fat in the liver in this instance was found, but even so, in 3 days, the value had dropped to that which is normal for extrauterine life.

We now proceeded to carry out the experiments which we originally intended to do; that is, to mobilize the fat in pregnant animals and to observe the changes thereby effected in the maternal and embryonic livers. Because of the normal infiltration of fat in the liver which takes place during the later period of gestation, animals were selected in which the embryos were below 40 gm. in weight. The mobilization of fat was effected in these animals by (1) fasting, (2) injection of phlorhizin, and (3) a combination of (1) and (2). At the end of the experimental period

TABLE IV.

Litter No.	Age of guinea pig.	Weight.	Higher fatty acids in liver.	Iodine value.
	hrs.	*gm.*	*per cent*	
1	16	62	18.49	101
	40	60	7.63	105
	64	79	6.33	104
2	12	78	13.5	104
	36	86	4.19	110
	60	87.5	3.97	128
3	16	98	9.09	106
	38	91.5	3.89	106
4	20	54	13.56	110
	56	88	5.53	108
5	30	63	7.33	107
	54	59	4.88	106
	80	85	3.38	111
6	30	85	5.17	109
	48	70	3.40	100
7	24	78	15.50	110
	48	70	5.93	114
8	16	67	25.60	88
	40	74	15.97	91
	80	77.5	2.53	108

the animals were killed and the fat determinations made as in the previous experiments. Protocols of the experiments are now given.

Experiment 1.—Pregnant guinea pig. Weight 610 gm. Received three doses of 0.05 gm. of phlorhizin subcutaneously at intervals of 24 hours. During the whole period the animal was fasted but allowed water.

Weight at the end of the period 450 gm. Weight of each embryo 5 gm.

The liver of a normal embryo at this age has the characteristic reddish color of normal hepatic tissue but in the embryos of this experiment the liver was pale and yellow; in fact it resembled the liver seen in the embryo during the last week or so of gestation.

Embryonic liver, higher fatty acids 9.39 per cent, iodine value 93.
Maternal " " " " 2.59 " " " " 131.

Experiment 2.—Pregnant guinea pig fasted for 3 days.

Received no phlorhizin. Average weight of embryos 8 gm.

Embryonic liver, higher fatty acids 9.65 per cent, iodine value not obtained.

Maternal liver, higher fatty acids 3.04 per cent, iodine value not obtained.

Experiment 3.—Pregnant guinea pig treated exactly as in Experiment 1.

Normal weight 765 gm. Weight at end of period 643 gm.

Embryonic liver, higher fatty acids 13.72 per cent, iodine value 95.
Maternal " " " " 7.18 " " " " 107.

Experiment 4.—Pregnant guinea pig treated as in Experiment 1.

Normal weight 685 gm. Weight at end of period 542 gm.

Weight of embryo 14.5 gm.

Embryonic liver, higher fatty acids 9.47 per cent, iodine value 94.
Maternal " " " " 3.44 " " " " 122.

Experiment 5.—Pregnant guinea pig received 0.05 gm. of phlorhizin subcutaneously on three occasions at intervals of 24 hours. No fasting. Weight of embryo 25 gm.

Embryonic liver, higher fatty acids 5.37 per cent, iodine value 108.
Maternal " " " " 1.80 " " " " 120.

Experiment 6.—Pregnant guinea pig, fasted for 96 hours, received no phlorhizin.

Normal weight 575 gm. Weight at end of period 420 gm.

Weight of embryo 25 gm.

Embryonic liver, higher fatty acids 9.63 per cent, iodine value 95.
Maternal " " " " 2.75 " " " " 131.

Experiment 7.—Pregnant guinea pig treated as in Experiment 1.

Normal weight 640 gm. Weight at end of period 528 gm.

Weight of embryo 30 gm.

Embryonic liver, higher fatty acids 9.17 per cent, iodine value 86.
Maternal " " " " 4.29 " " " " 122.

Experiment 8.—Pregnant guinea pig treated as in Experiment 1, w the exception that it received but two doses of phlorhizin.

Normal weight 817 gm. Weight at end of period 666 gm.

Weight of embryos 30 gm.

Determinations were made upon each of the two embryos.

Embryonic liver (1), higher fatty acids 13.78 per cent, iodine value 95; (2), higher fatty acids 18.00 per cent, iodine value 94.

Maternal liver, higher fatty acids 2.80 per cent, iodine value 130.

The results of these experiments are given in Table V and are arranged in the order of the increasing weight of the embryos.

TABLE V.

Weight of embryo.	Embryonic liver.		Maternal liver.	
	Higher fatty acids.	Iodine value.	Higher fatty acids.	Iodine value.
gm.	*per cent*		*per cent*	
5.0	9.39	93	2.59	131
6.0	9.69		3.04	
8.0	13.72	95	7.18	107
14.5	9.47	94	3.44	122
25.0	5.37	108	1.80	120
25.0	9.63	95	2.75	131
30.0	9.17	86	4.29	122
30.0	13.78	95	6.49	103
30.0	18.00	94	6.49	103

The results of these experiments show that in all the embryos a marked fatty infiltration of the liver was produced. Even at as early a stage in development as represented by a weight of 5 gm., the liver tissue takes up fat. In the first six series of figures in Table V all the embryos weighed less than 30 gm. and in only one of these instances was there an associated infiltration of fat in the maternal liver and in that particular instance the highest fat value in the embryonic liver was obtained. It is interesting to note the fall in the iodine value of the maternal liver fat in this instance.

There is a marked contrast in the iodine values of the embryonic and maternal liver fat; the former are much lower than the latter. The average iodine value of the embryonic liver fat in these cases is 95. The average fat content of the embryonic livers is 10.91 per cent. If, as in the case of the physiological infiltration of fat, in the embryonic livers, we assume for purposes of calculation that the fat present in excess of the average amount is of the connective tissue type with an iodine value of 85, we find that the iodine value thus obtained would be 91. The value actually found is 95. This result, then, agrees closely with the theoretical value and much more so than the result obtained in the case of the physiological and slower infiltration of development. This seems to show that under the experimental conditions the infiltrated fat is of the connective tissue type and as such accumulates in the liver of the embryo.

Experiments similar to those just described were carried out upon pregnant guinea pigs in which the embryos were over 40 gm. in weight, and in which, therefore, the physiological infiltration had been established. Protocols of two experiments are given.

Experiment 9.—Pregnant guinea pig treated as in Experiment 1.

Normal weight 905 gm. Weight at end of period 756 gm.

Weight of embryos 56 gm.

Embryonic liver (1), higher fatty acids 19.39 per cent, iodine value 86; (2), higher fatty acids 20.60 per cent, iodine value 82.

Maternal liver, higher fatty acids 6.49 per cent, iodine value 104.

Experiment 10.—Pregnant guinea pig treated as in Experiment 1.

Normal weight 826 gm. Weight at end of period 725 gm.

Weight of embryos 53 and 56 gm.

Embryonic liver (1), 53 gm., higher fatty acids 30.49 per cent, iodine value 87; (2), 56 gm., higher fatty acids 24.12 per cent, iodine value 88.

Maternal liver, higher fatty acids 2.74 per cent, iodine values 122.

These two experiments show that the physiological infiltrated fat does not represent a maximum capacity. This is strikingly brought out in Experiment 10 where a value of 30.49 per cent was obtained, and this fat, it should be remembered, is calculated upon the moist tissue. In this instance it is also of interest to note the absence of any infiltration of fat in the maternal liver.

DISCUSSION.

The accumulation of the fat in the livers of the embryo guinea pigs during the latter part of gestation, the unsaturated nature of the fat, and its rapid disappearance after birth suggest that it is material required by the young animal after its communication with the maternal circulation has ceased. It is, however.

possible that this fat is not utilized for the production of heat and energy but is transferred to the connective tissues after being converted into fat of the connective tissue type.

The question arises whether this fatty infiltration of the liver is limited to guinea pigs or whether it takes place in other animals such as the dog, cat, or rabbit. Observations upon this point have as yet not been made. The guinea pig at birth is quite a mature animal and is very active immediately after birth, and these characteristics may in some way be associated with the fat in the liver and its rapid disappearance.

What is the source of the fat which accumulates in the liver during fasting? Is it derived from the embryonic or maternal connective tissue fat? It is at present impossible to say. Some light might be thrown upon the subject by a determination of the fat in the embryo after the removal of the liver.

Fasting produces a fatty infiltration of the embryonic liver much more readily than in the maternal liver. If the fat were maternal in origin, it would indicate an avidity for fat on the part of the embryonic liver.

SUMMARY.

1. In the latter part of embryonic life, it is normal for the fetal liver in guinea pigs to contain large amounts of fat.
2. This fat has an iodine value as high as is normal in later life when a much smaller amount of fat is present.
3. This fat decreases in the first 48 to 72 hours after birth and then no more is found in the liver than later in life.
4. If fat is mobilized experimentally by phlorhizin and fasting in pregnant guinea pigs, with embryos under 40 gm. in weight, fat accumulates in the liver of the embryos, but the fat is of the connective tissue type.
5. When the embryos are above 40 gm. in weight, and the fat is mobilized, more fat is found in the liver than otherwise, up to 30 per cent of the fresh tissue, and this fat too is of the connective tissue type.
6. The infiltration of fat in the embryonic liver may be quite independent of any similar change in the maternal liver.

It is a pleasure to acknowledge our thanks to Professor J. B. Leathes, Sheffield, for his review and kindly criticism of this work.

BIBLIOGRAPHY.

1. Leathes, J. B., The fats, London, 2nd edition, 1913.
2. Wys, J. J. A., *Ber. chem. Ges.*, 1898, xxxi, 750.
3. Imrie, C. G., *J. Path. and Bact.*, 1914-15, xix, 245.
4. Leathes, J. B., and Meyer-Wedell, L., *J. Physiol.*, 1909, xxxviii, p. xxxviii.

SOME EFFECTS OF WATER-SOLUBLE VITAMINE UPON NUTRITION.*

BY WALTER G. KARR.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, August 4, 1920.)

Recent studies have shown the peculiar rôle of certain o called accessory substances or vitamines in nutrition. The bsence of these factors affects materially the physiological well eing of the animal and in some cases leads to a disease typical or the lack of the specific accessory substance. The data on vhich such conclusions are based have for the most part been ecured from observations on small laboratory animals; *viz.*, irds, rats, mice, and guinea pigs. No broad generalizations can e made, however, until the problem of the accessory factors has een studied on more species of animals, including the larger nes. This is well illustrated by the fact that a diet which will eadily induce scurvy in a guinea pig will not produce the disease n a rat with the same readiness, if at all. This study is a contribution to the relation of the so called water-soluble B vitamine o the nutrition of the dog, with the metabolism of which we are airly well acquainted.

Dogs were kept upon standard diets containing either wheat gluten[1] or commercial casein[2] as the source of protein, with the emaining calorific requirement supplied by lard and sucrose. It is quite probable that these sources of protein contained traces

---

* The data in this paper are taken from the dissertation presented by the author for the degree of Doctor of Philosophy, Yale University, 1920.

Part of the expenses of this investigation were defrayed by a grant from the Russell H. Chittenden Research Fund for Physiological Chemistry.

[1] Purchased from the Pure Gluten Food Company, Columbus, Ohio.

[2] Purchased from Lister Brothers, Incorporated, New York.

of water-soluble vitamine, although the diets are referred to in the present paper as being vitamine-free.[3]

Foods.—The compositions of the food mixtures, representing approximately that quantity which was given each animal daily per kilo of body weight, were as follows:

No. 1.

	gm.	
Commercial casein (12.7 per cent N)...	6.3	Contained 0.8 gm. N and 80 calories.
Sucrose	4.5	
Lard	4.1	
Bone ash	0.4	
Salt mixture	0.2	
Water	2.5	
Total	18.0	

No. 2.

	gm.	
Commercial gluten (14.0 per cent N)...	5.7	Contained 0.8 gm. N and 80 calories.
Sucrose	4.6	
Lard	4.3	
Bone ash	0.4	
Salt mixture	0.2	
Water	3.8	
Total	19.0	

No. 3..

	gm.	
Commercial casein (12.7 per cent N)...	3.1	Contained 0.4 gm. N and 80 calories.
Sucrose	5.2	
Lard	5.2	
Bone ash	0.4	
Salt mixture	0.2	
Water	0.8	
Total	15.0	

The salt mixture contained: sodium chloride, 10 gm.; calcium lactat 4 gm.; magnesium citrate, 4 gm.; ferric citrate, 1 gm.; a few drop I+KI solution.

---

[3] It will be observed that the food used in the following experiment are also practically free from the so called fat-soluble vitamine and th vitamine preventing scurvy. While the animals have been under obse vation there have been no symptoms which might be taken as specificall indicative of the lack of these vitamines.

The foods were made up in fairly large quantities and the calculated amounts fed in a single meal each morning. The ration was left before the animals for a period of 1 to 3 hours whereupon any residue was weighed.

Vitamine Preparations.—The following sources of the so called water-soluble B vitamine were used in the experiments:

A. Brewery Yeast.—A dried commercial product.[4] Nitrogen, 7.2; moisture, 9.1; ash, 6.6 per cent.

B. Autoclaved Brewery Yeast.—Some of Product A was moistened and autoclaved for 3 to 4 hours at 20 pounds pressure, then dried in the hot air oven at 108°C. for 72 hours. Nitrogen, 7.8 per cent.

C. Redried Brewery Yeast.—Some of Product A was moistened and dried in the hot air oven at 108°C. for 72 hours. Nitrogen, 8.2 per cent.

D. Bakers' Yeast.—A moist commercial yeast[5] was dried in the hot air oven at 108°C. for 72 hours. Nitrogen, 6.1 per cent. This preparation contained an admixture of starch.

E. Milk.—A pasteurized dairy milk, some of which was further sterilized in the laboratory by heating in a boiling water bath for 30 to 60 minutes.

F. Tomatoes.—Canned tomatoes were squeezed as much as possible through cheese-cloth so as to produce a fine suspension. Nitrogen, 0.14 per cent; solids, 4.5 per cent.

G. Concentrated Vitamine Extract.—A crude preparation was made according to the directions of Osborne and Wakeman.[6] The fraction precipitated by 80 per cent alcohol was reprecipitated and dissolved in water. That obtained from 165 gm. of Yeast A was dissolved in 200 cc. of water. Nitrogen, 0.52 per cent.

EXPERIMENTAL.

Most of the dogs consumed the diet readily at first, subsequently they refused a part, and ultimately failed to eat any whatever of the mixtures unless vitamine-containing food was given. A number of the animals which continued to ingest the vitamine-free foods for a longer period developed pathologic symptoms.

Water-Soluble B Vitamine and Food Intake.—In all animals which refused the whole or part of their diet the desire to eat could be quickly restored by the ingestion of a substance containing water-soluble vitamine. The plan of the experiments was to feed the vitamine-free food until the dog refused part or the whole of it for a number of days, then feed the accessory substance and note the effect on the food intake. The preparation of water-soluble vitamine was given apart from the food after the meal had been offered in the morning, hence it could not affect the palatability of the ration.

---

[4] Supplied by Hinckel Brewery, Albany, N. Y.

[5] Marketed by Fleischmann Company, New York.

[6] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

Charts 1 to 29 illustrate the relation of the vitamine to food intake. The maximum ordinate represents the food in grams offered to the dog to eat. In the shaded portion is plotted its actual daily food intake.

Brewery Yeast as the Source of Vitamine.

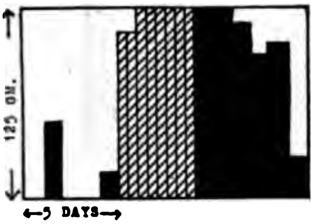


Chart 1. Dog X, days 24 to 38, Food 3.

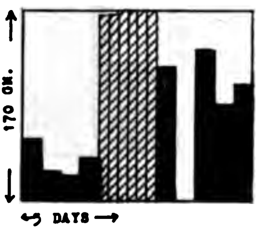


Chart 2. Dog B, days 39 to 50, Food 1.

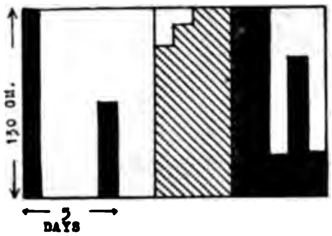


Chart 3. Dog S, days 20 to 35, Food 3.

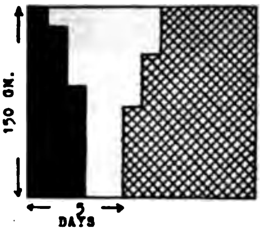


Chart 4. Dog F, days 19 to 30, Food 1.

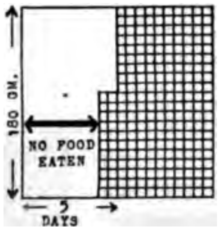


Chart 5. Dog H, days 22 to 31, Food 1.

■ DIET
▨ DIET 1/2GM. YEAST A
▧ DIET + 1GM. YEAST A
▩ DIET + 3GM. YEAST A
▦ DIET+4GM. YEAST A

Bakers' Yeast as the Source of Vitamine.

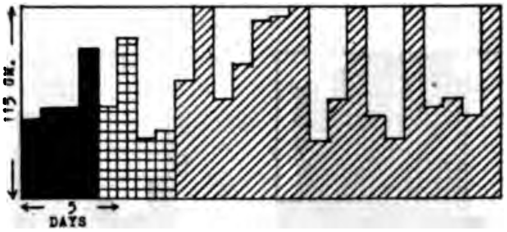


Chart 6. Dog R, days 27 to 51, Food 3.

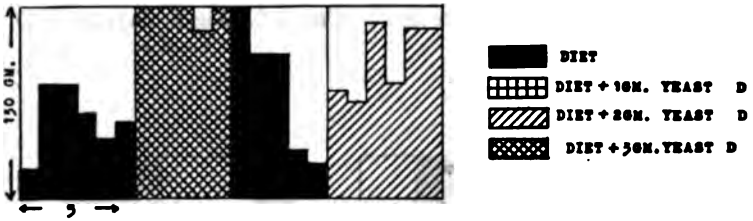


Chart 7. Dog I, days 101 to 122, Food 1.

Tomato as the Source of Vitamine.

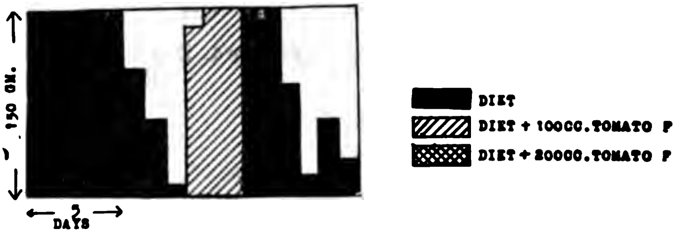


Chart 8. Dog I, days 141 to 157, Food 1.

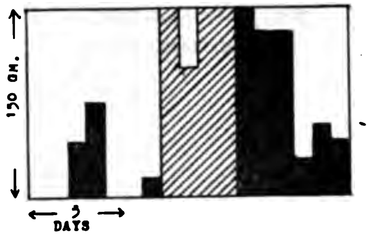


Chart 9. Dog P, days 41 to 57, Food 2.

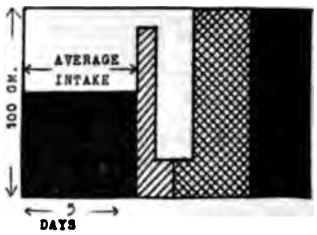


Chart 10. Dog J, days 143 to 157, Food 1.

Milk as the Source of Vitamine.

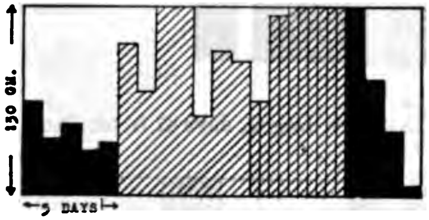


CHART 11. Dog I, days 235 to 255, Food 1.

■ DIET
▨ DIET + 50CC. MILK E
▩ DIET + 75CC. MILK E
⊞ DIET + 100CC. MILK E

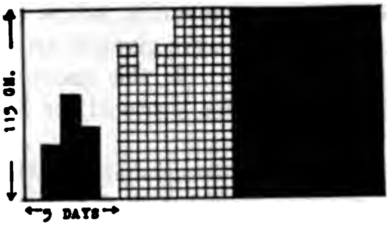


CHART 12. Dog J, days 224 to 242, Food 1.

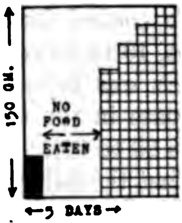


CHART 13. Dog L, days 179 to 187, Food 1.

To eliminate the possibility that this phenomenon of renewed appetite might be due to the supplementing action of the protein in the vitamine preparation, experiments were conducted with a concentrated vitamine extract.

Experiments with Concentrated Extract.

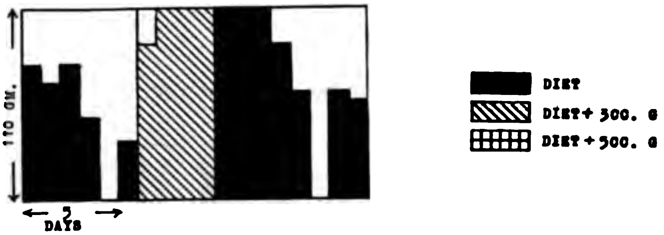


Chart 14. Dog Q, days 41 to 58, Food 1.

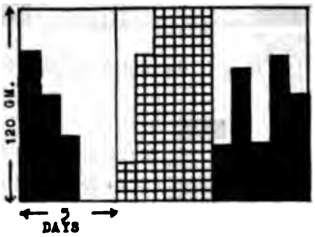


Chart 15. Dog O, days 92 to 106, Food 2.

To control further the possible supplementing action of proteins, 10 to 15 gm. of casein diet, No. 1, were given to an animal which was being offered gluten diet, No. 2; this provoked no increase in the food intake (see Appendix, protocol for Dog O, days 90 to 95).

That the failure of appetite is actually associated with the lack of vitamine is further shown by the fact that it does not occur when the accessory food factor is furnished from the very start along with the vitamine-free food. Dog L (see Appendix, protocol for Dog L, days 1 to 120) was placed on casein diet, No. 1, to which were added 3 gm. of Yeast A daily. This animal continued to eat throughout a period of 120 days, was in good health, and gained weight when the diet was increased in amount. The longest period that any animal continued on the same diet without vitamine was 68 days (see Appendix, protocol for Dog G, days 1 to 68).

Two animals were fed quantities of 10 to 15 gm. of butter (see Appendix, protocols for Dogs L and I) to ascertain whether or not the fat-soluble vitamine might influence the appetite. No appreciable increase in the food intake was noted.

Experiments on the Destruction of the Vitamine.—To determine the influence of drying the vitamine preparation at temperatures near 100°C. experiments were conducted with Yeast C (Charts 16, 17, 18).

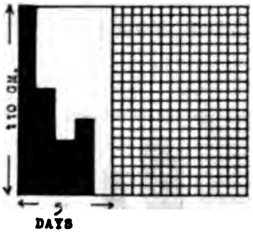


Chart 16. Dog Q, days 82 to 93, Food 1.

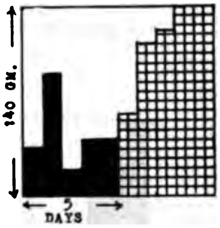


Chart 17. Dog P, days 74 to 83, Food 2.

■ DIET
▦ DIET+½ GM. YEAST C
▧ DIET+1 GM. YEAST C
▨ DIET+1½ GM. YEAST C

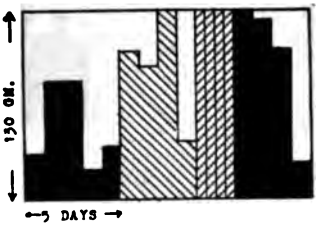


Chart 18. Dog I, days 197 to 224, Food 1.

These data indicate that little or no vitamine was destroyed by drying at temperatures near 100°C.

To determine the influence of still higher temperatures Yeast B was fed (Charts 19, 20, 21, 22).

Some of the accessory substance had apparently been destroyed, as it required considerably more of this preparation to provoke the ingestion of the regular ration.

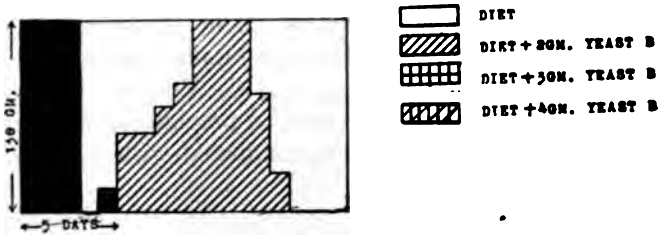


CHART 19. Dog E, days 86 to 102, Food 1.

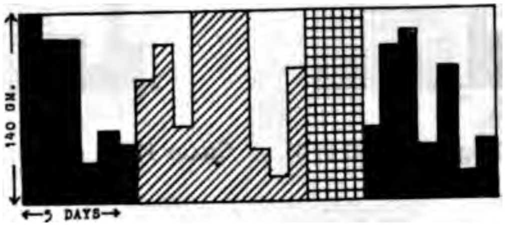


CHART 20. Dog P, days 53 to 77, Food 2.

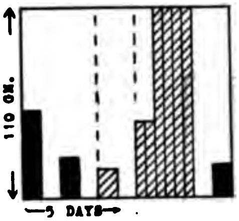


CHART 21. Dog J, days 267 to 277, Food 1.

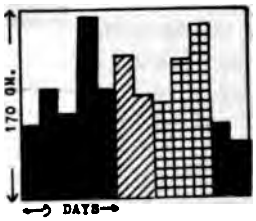


CHART 22. Dog B, days 63 to 74, Food 1.

Quantitative Relations.—1.5 gm. of Yeast A have in all cases been sufficient to bring the food intake back to normal, and often 0.5 gm. was sufficient, while with Yeast D 3 gm. were approximately the minimum which would produce this result. Charts 23 and 24 show a direct comparison between Yeasts A and D, while Chart 25 shows clearly the destruction of vitamine in Preparation B.

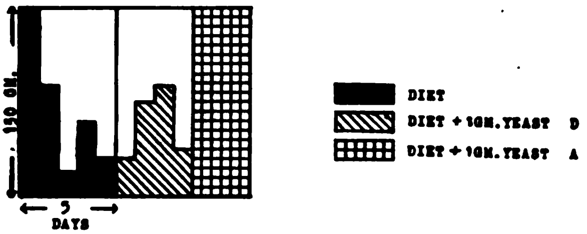


CHART 23. Dog I, days 153 to 164, Food 1.

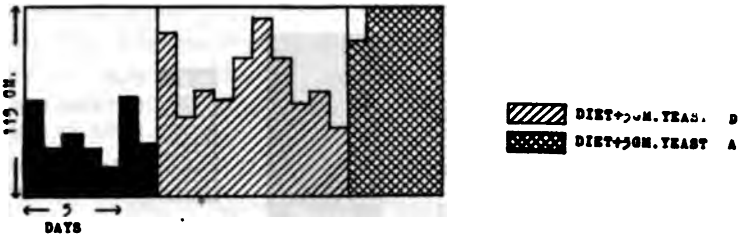


CHART 24. Dog J, days 73 to 94, Food 2.

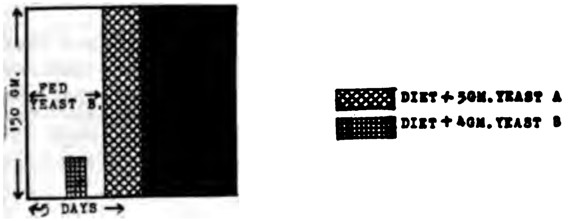


CHART 25. Dog L, days 151 to 161, Food 1.

100 cc. of tomato, Preparation F, were often effective while 200 cc. have not failed to restore the food intake. 50 to 150 cc. of milk have proved sufficient.

Charts 26, 27, 28, and 29 show the effect of gradually increasing amounts of vitamine preparation added to the diet until the animals consumed the total amount of food offered.

A rather definite minimum amount of water-soluble vitamine seems to be necessary to sustain the desire to partake of food

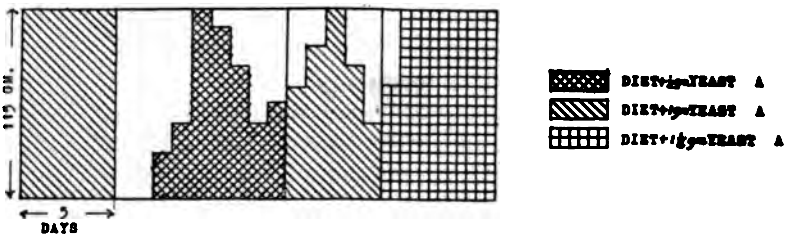


CHART 26. Dog J, days 102 to 126, Food 2.

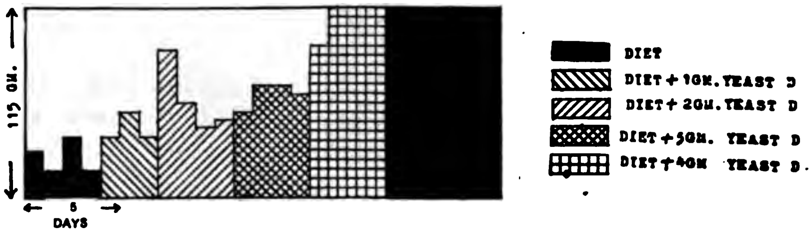


CHART 27. Dog J, days 192 to 216, Food 1.

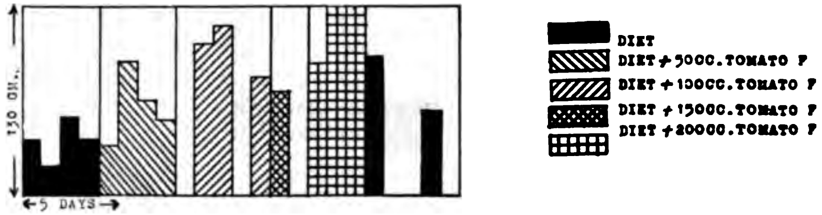


CHART 28. Dog S, days 34 to 56, Food 3.

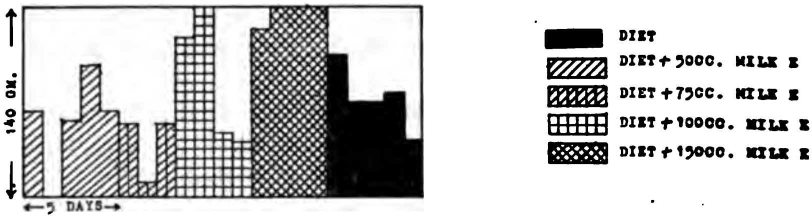


CHART 29. Dog P, days 144 to 159, Food 1.

which is otherwise adequate for the nutrition of the animal. The quantity varies with the animal. 0.5 gm. of Yeast A was sufficient for some animals while others required 1.5 gm., and in a similar way the requisite amounts of the other vitamine preparations vary. The needs for the same animal seem to be quite constant. Dog J, which was refusing most of the gluten diet, No. 2, offered, was given 3 gm. of Yeast D. This increased the food intake, but was not quite adequate to cause the ingestion of all the food. 120 days later when the animal was on a casein food and in better nutritive condition it was again given 3 gm. of Yeast D. This amount again was not sufficient to cause the ingestion of the entire diet, but, following this period, 4 gm. were found to be effective. The quantitative requirements of this animal were very similar under widely different circumstances (see Charts 24 and 27). Furthermore, animals which require a larger dosage of one vitamine preparation will require correspondingly larger dosages of the other preparations. Amounts of vitamine not adequate to cause the ingestion of all the offered food, nevertheless, generally increase the food intake perceptibly.

In two cases (see Appendix, protocols for Dog H, days 82 to 86, and Dog E, days 66 to 75) the animals were allowed to remain in a state of inanition and became very emaciated. Attempts to resuscitate the desire of these animals to eat failed even though large quantities of yeast were given. One recovered when milk was given by sound; the other was not given milk and died.

Pathologic Symptoms of Vitamine Deficiency.

In certain animals which continued to eat their food for a longer time than others without the addition of any accessory substance, there appeared typical pathologic symptoms (see Appendix, protocols for Dogs F, G, I, M, R). The animals first vomited, became inactive, refused their food, and then developed characteristic polyneuritic symptoms. The animals could not walk, finally could not stand, and generally lay with their legs sprawled out at the side. They were seized with severe tetanic convulsions especially when handled, removed from the cage, or set down quickly on the floor. The head was in motion and there were often tremors and twitchings of the muscles. These symptoms are comparable with those described by Voegtlin and Lake.[7] Either the loss of control of the legs or the convulsions may appear first. The above authors do not mention the preliminary vomiting and they lay stress on the loss of body weight which from the present data seems only concomitant with the lowered food intake, as some of the animals maintained their weight until they refused the food, a short time before the polyneuritic symptoms became apparent (see protocols for Dogs G, M, R). Dog I which had a lowered food intake grew very emaciated before developing the symptoms.

These pathologic symptoms could be alleviated and practically eliminated within a period of a few hours by administering water-soluble vitamine in the form of brewery yeast or tomato. Dog G which had the most severe convulsions, numerous and occurring frequently, and had no use of its legs, was given 5 gm. of Yeast A and when observed 8 hours later was able to run from its cage with a slight loss of control of the hind legs as the only apparent symptom remaining. Dog S, having severe convulsions, was given 3 gm. of Yeast A which was sufficient to eliminate all polyneuritic symptoms and cause the animal to consume all its food for a number of days afterwards. 40 days later the animal again became polyneuritic and was cured by administering 400 cc. of tomato, Preparation F. After 60 days the animal developed the third attack of polyneuritis. It was fed 10 gm. of Yeast D, and showed no improvement the following day when it was given a 20 gm. dose. The animal died during the night. It is very probable that the bakers' yeast was much less efficacious in alleviating polyneuritic symptoms than equivalent amounts of brewery yeast. Dog F was given doses of 3 gm. of Yeast A without any beneficial result and later died. The dog was at the time nursing three young which might have been a sufficient drain on the mother's source of vitamine so that the dose of 3 gm. produced no curative effect. This dog only showed paralytic symptoms of the legs and had no convulsions. Dog G at the second attack of the disease was given a dose of 3 gm. of Yeast A without any alleviation of the symptoms and was given 10 gm. the next day when its condition was so bad that no hope of recovery was entertained. Its temperature at this time was 28°C. Dog M differed from the other animals in that it died rather suddenly before having exhibited many of the characteristic symptoms.

---

[7] Voegtlin, C., and Lake, G. C., *Am. J. Physiol.*, 1918–19, xlvii, 558.

SUMMARY AND CONCLUSIONS.

Some relation exists in the dog between the desire to partake of food and the amount of the so called water-soluble B vitamine ingested.

Brewery yeast, bakers' yeast, tomatoes, and milk were investigated and found to be sources of this appetite-provoking substance. Quantitative data are presented to show the relative potency of the various products investigated. The brewery yeast tested was much more potent than bakers' yeast.

Drying at 100°C. does not affect the efficacy of these vitamine preparations, but autoclaving at 120°C. for 3 to 4 hours leads to some destruction.

Mammalian polyneuritis was induced in several animals and the symptoms were alleviated within a few hours by the ingestion of brewery yeast or tomatoes, substances rich in water-soluble vitamine.

The writer desires to express his gratitude to Professor Lafayette B. Mendel for his suggestion of the problem and for his generous advice and interest throughout the course of this investigation.

APPENDIX.

Dog B.—Offered 170 gm. of Food 1 daily.

Ate food for the first 24 days whereupon the food intake diminished. Days 42 to 45. Fed brewery Yeast A (see Chart 2). Days 67 to 73. Fed autoclaved Yeast B (see Chart 22).

Body weights:

Day.......	1	8	15	22	29	36	45	52	63
Kilos......	11.5	10.7	10.9	10.7	10.7	10.2	10.0	10.0	9.7

Dog E.—Offered daily 110 gm. of Food 1.

During the first 8 days the animal was given 3 gm. of Yeast A in addition to its food, after which it continued to eat its food and nourish a litter of three pups until the 33rd day when it began to refuse part of its food. It continued on this lowered food intake until the 66th day whereupon it had become very emaciated. It was given on 4 successive days, 5 gm. of Yeast D, 10 gm. of Yeast D, 5 gm. of Yeast A, and 5 gm. of Yeast A, which provoked no ingestion of food. It was then fed 250 cc. of milk for 3 days and on the following day ate the regular diet. The diet was increased to 130 gm. On the 89th day it again refused its food whereupon it was fed 2 gm. of Yeast B daily which provoked the food intake represented in Chart 19. The animal died on the 120th day and the autopsy showed diseased lungs.

Body weights:

Days...	8	20	34	42	48	57	62	67	75	80	87	94	119
Kilos...	6.6	5.1	4.7	4.6	4.6	4.2	3.9	3.6	3.3	3.7	3.9	3.7	3.3

Dog F.—Offered daily 180 gm. of Food 1.

Ate well for the first 18 days whereupon she gave birth to four young. She then refused part of her food and on the 22nd day ate none. Fed 3 gm. of Yeast A. Consumed regular diet (see Chart 4). Continued to eat and was fed 3 gm. of Yeast A daily during days 30 to 35. On the 36th day the dog lost control of its hind legs and could hardly stand. It was given 3 gm. of Yeast A in a.m. and 3 gm. more in p.m. On the 37th day the dog was about the same in a.m. but worse in p.m. It lay with its legs sprawled out at the side and could not raise its head. It was able to wag its tail and was in no apparent pain. On the morning of the 38th day the dog was found dead. (Dog had a marked polyuria during the whole period under observation.)

Body weights:

Days........................	1	7	15	19	25	31
Kilos........................	10.0	9.6	9.6	8.8	7.6	7.7

Dog G.—Offered 140 gm. of Food 1.

Dog ate food well until the 63rd day when it vomited a portion. It vomited its food for 3 or 4 days and on the 67th day lay down most of the time. On the 68th day the dog for the first time did not eat its food. It spasmodically lost control of its legs when they shot out at the side of the body and the head was drawn to one side. On the 69th day its condition was about the same and it continued to refuse food. The next day the animal sat with its hind legs sprawled out at the side and could not walk. 71st day. Head shook; some tremors of the leg muscles; spasmodic tetanic convulsions when dog straightened the fore legs out front and the hind legs to the rear with the head thrown back. This occurred especially when the dog was set on the floor. 11:30 a.m. was given 5 gm. of Yeast D by sound. 3 p.m. the dog was much worse. The convulsions were more severe, the dog throwing itself over on its side and banging its head on the floor. These occurred every 2 or 3 minutes while the dog was under observation outside the cage. 3:30 p.m. given 5 gm. of Yeast A by sound. 12 midnight, dog ran from the cage. All nervous symptoms were gone except that it did not have full control of its hind legs, a condition which persisted for a few days. The next day the dog ate all its food. On the 98th day the vitamine was again removed. The dog continued to eat well again until the 145th day when it refused its food. It continued to refuse its food for 5 days when polyneuritic symptoms began to be noted. It had some convulsions and walked with difficulty. 151st day. 1 p.m., the dog was given 3 gm. of Yeast A. 152nd day. The dog was much worse; temperature 28.3°C. It was given 10 gm. of Yeast A in the a.m. but the animal's condition was so bad that no hope of recovery was entertained and it died during the night.

Body weights:

Day	1	7	13	22	28	34	40	46	52	59
Kilos	8.1	7.8	7.6	7.4	7.2	7.2	7.4	7.1	7.3	7.2

Day	67	71	74	77	81	84	91	103	109
Kilos	6.9	6.3	6.6	6.9	6.9	6.8	6.9	6.8	7.1

Day	116	123	136	142	151
Kilos	7.2	7.2	7.2	7.4	6.3

Dog H.—Offered 185 gm. of Food 1 daily.

The dog ate well for 8 days, refused part of food for 9 days, and ate little for 7 days when it refused food. 25th day. Fed 4 gm. of Yeast A (see Chart 5). Yeast was discontinued on the 36th day. Ate all until the 47th day and from then until the 80th day generally ate a small portion of its food. The animal became at this time very emaciated without developing any polyneuritic symptoms and was evidently in a starving condition. Attempts were made to cause it to eat some food by feeding on successive days: 3 gm. of Yeast D, 10 gm. of Yeast D, 5 gm. of Yeast A, 10 gm. of Yeast A, 10 gm. of Yeast A; but the dog refused to eat and died on the 86th day. Inanition had evidently proceeded too far for the yeast to influence the appetite.

Body weights:

Day	1	7	13	19	25	31	37	43	49
Kilos	12.0	11.6	11.3	10.4	9.6	10.0	10.3	10.6	10.9

Day	56	63	71	77	84
Kilos	9.9	9.3	8.8	8.5	7.6

Dog I.—Offered 110 gm. of Food 1 daily.

Ate food well for 20 days. For the next 25 days it ate a portion of its food each day with an averge food intake of 55 gm. 45th day. The dog could hardly stand, the hind legs more paralyzed than the fore; the head was in motion from side to side. 10 a.m. it was fed 5 gm. of Yeast A, 5 p.m. much improved and ran from the cage with only a slight wobble in its hind legs. The dog was in poor nutritive condition and was fed yeast with an increased diet in order that it might gain weight. 71st day. The yeast was discontinued and the food offered daily was now 130 gm. of No. 1. 85th to 105th days. Ate only part of its food. 106th to 123rd days. Experiments were conducted with bakers' yeast (see Chart 7). 147th day. Again refused part of its food. Experiment was conducted with tomatoes as an accessory (see Chart 8). This was followed by an experiment in which 1 gm. of Yeast D was compared with 1 gm. of Yeast A (see Chart 23). The dog again in poor nutritive condition was given an increased diet with 2 gm. of Yeast A daily. It was placed on the regular diet at the 180th day. For the next period of 13 days the animal was given 10 to 15 gm. of butter daily in order to determine whether or not the fat-soluble vitamine might have any effect on the appetite. No appreciable increase in the food intake was observed. 200th day. Accessory Preparation C was tried (see Chart 18). The animal was without accessory from the 208th to the 239th day when experiments with Milk E as accessory were tried (see Chart 11). Days 251 to 264. No accessory. 265th day. Fed 2 gm. of Yeast C which caused it to consume all its food for 4 days. Days 272 to 275. Fed Yeast B.

Body weights:

Day........	1	7	19	25	31	37	45	52	59	67
Kilos.......	6.9	6.4	6.3	5.8	5.4	5.1	4.5	5.1	5.1	5.5

Day.......	74	81	88	98	106	113	120	133	142
Kilos......	5.9	6.1	5.8	5.3	4.9	5.2	4.7	4.6	4.9

Day...	148	155	162	169	176	183	190	197	204
Kilos..	4.8	4.8	4.6	5.2	5.7	5.4	5.5	5.3	5.3

Day..........	211	218	225	232	239	248	256	267
Kilos.........	5.4	5.7	5.5	5.6	5.3	5.5	5.5	5.2

Dog J.—Offered 115 gm. of Food 2 (gluten) daily.

First 75 days the animal was used for metabolism experiments and had received Accessory A on certain days. At the end of the period it was in rather poor nutritive state. 73rd to 94th days. See Chart 24. 102nd to 126th days. See Chart 26. 125th to 133rd days. Yeast discontinued, eats part of food. 134th day. Changed to 100 gm. of Food 1. After this change of protein the dog consumed the whole diet for 6 days after which food intake decreased. 143rd to 157th days. See Chart 10. It was now supplied with all the food that it would eat together with 2 gm. of Yeast A daily in order that it might gain weight. 177th day. Placed on diet of 110 gm. of Food 1. 192nd to 215th days. See Chart 27. 223rd to 240th days. See Chart 12. From this period on it was continued on the same diet and used for other experiments. Days 270 to 275. Fed Yeast B (see Chart 21).

Body weights:

Day	1	7	13	19	25	31	37	47	52	59
Kilos	6.6	6.4	6.2	6.2	5.8	5.4	5.5	5.5	5.7	5.5

Day	67	74	81	88	96	103	109	116	123
Kilos	4.8	4.5	4.4	4.2	4.4	4.5	4.2	4.2	4.1

Day	133	142	148	155	162	169	176	183
Kilos	4.0	4.1	3.8	4.5	4.7	5.0	5.7	5.5

Day	190	197	204	211	218	225	232	239
Kilos	5.7	5.2	5.1	5.3	5.3	5.2	5.5	5.5

Day	248	256	267
Kilos	6.2	5.9	5.8

Dog L.—Offered 130 gm. of Food 1 daily.

1st to 120th days. Given 3 gm. of Yeast A daily. It ate the food very readily for the first 90 days. During the last 30 days it seemed to tire of the diet to some extent and would occasionally leave a small amount of its food. It was a rather young dog and between the 30th and 80th days its diet was increased to 180 gm. with the resultant increase in body weight. Dog was now offered 150 gm. of Food 1 daily. During the next 20 days the food intake gradually declined to a small amount each day. 140th to 147th days. Given 10 to 15 gm. of butter to determine the effect of the fat-soluble vitamine. The food intake was not increased. 148th to 156th days. See Chart 25. 156th to 181st days. Food intake decreased with no accessory added. 182nd to 186th days. See Chart 13. The dog had developed a skin disease and was killed.

Body weights:

Day	1	8	15	22	28	35	42	49	54	60
Kilos	6.6	6.7	6.6	6.6	6.6	6.8	6.9	6.9	6.9	7.2

Day	67	74	84	93	99	106	113	120	127
Kilos	7.4	7.6	7.6	7.9	7.8	8.0	8.1	8.3	8.3

Day	134	141	148	155	162	169	176	183
Kilos	8.3	7.7	7.3	7.3	7.5	7.8	7.4	7.4

Dog M.—Offered 125 gm. of Food 1 daily.

Ate whole diet for 21 days after which the food intake was more or less decreased until the 61st day when the dog vomited some for 2 days. 63rd day. Refuses its food. During the next 3 days symptoms of polyneuritis developed. It walked with difficulty; the head shook some. 67th day. Slight convulsions. Condition not considered very serious and was not treated with accessory. Died during the night.

Body weights:

Day	4	11	19	25	32	39	49	54	60	64
Kilos	6.5	6.5	6.4	6.3	6.4	6.2	6.3	5.9	6.2	5.4

Dog O.—Offered 130 gm. of Food 2 daily.

First 50 days was used for other experiments. 50th to 89th days. Food intake decreased on no accessory. 90th to 95th days. Fed 10 to 15 gm. of Food 1. There was no increase in food intake. 96th to 100th days. See Chart 15. The dog died on the 107th day. The autopsy showed no gross abnormalities except feces very tightly packed in the large intestine.

Body weights:

Day	54	64	71	74	84	93	99	106
Kilos	5.4	5.0	4.7	4.7	4.7	4.6	4.5	4.5

Dog P.—Offered 150 gm. of Food 2 daily.

1st to 33rd days. Ate the whole diet offered. 34th to 46th days. Food intake diminished near the end. 41st to 57th days. See Chart 9. 58th to 75th days. See Chart 20. 76th to 83rd days. See Chart 17. 84th to 100th days. Appeared sick and ate only a part of its food. 101st day. Diet was changed to 140 gm. of Food 1. 101st to 143rd days. Ate part of its food whereupon milk experiments were tried on 144th to 159th days (see Chart 29). 160th to 165th days. Food intake dropped whereupon Yeast B was fed. The dog was removed from the diet on the 173rd day in good condition.

Body weights:

Day	1	5	12	19	29	38	44	51	58	65
Kilos	7.8	7.9	7.6	7.3	7.1	7.2	6.7	6.7	6.3	6.3

Day	72	93	100	107	114	121	128	135
Kilos	6.3	6.1	6.1	6.0	6.3	6.2	6.0	6.0

Day	144	152	163
Kilos	5.8	5.6	5.9

Dog Q.—Offered 110 gm. of Food 1 daily.

Animal ate food for the first 20 days when the food intake diminished. 46th to 49th days. Yeast G was given (see Chart 14). 86th to 90th days. Redried brewery Yeast C was fed (see Chart 16). The dog later refused to eat and died on the 99th day. The cause of death was unknown.

Body weights:

Day	1	5	12	19	29	38	44	51	58	65
Kilos	5.3	5.4	5.3	5.4	5.2	5.2	5.1	5.4	5.1	4.9

Day	72	79	86	93
Kilos	4.5	4.8	4.6	5.0

Dog R.—Offered 115 gm. of Food 3 daily.

The animal was coaxed to eat the food for the first few days by adding a little milk. It ate the food until the 21st day when the food intake decreased. 30th to 52nd days. Fed Yeast D (see Chart 6). It ate little on 58th to 60th days and on the 61st day developed polyneuritic symptoms. The dog had spasmodic convulsions but the legs were not paralyzed to any extent. On the next day the convulsions were worse, but the dog could still stand on its legs. It was fed 3 gm. of Yeast A in a.m. 63rd day. The polyneuritic symptoms were absent. 64th day. It ate one-half its food. 65th to 77th days. Ate whole of the diet without further addition of yeast. 78th to 103rd days. The dog was on a diminished food intake and again developed polyneuritic symptoms. 106th day. 400 cc. of Tomato F were given by sound but the dog vomited most of it soon and was not improved the next day when 400 cc. more were given which it retained. The dog had recovered by the following day except for a slight stiffness of the legs which remained for a few days. It consumed all its food for 9 days and then the food intake diminished. 170th day. It again showed polyneuritic symptoms. 171st day. It was given 10 gm. of Yeast D. The next day it showed no improvement and was given 20 gm. of Yeast D. The next morning the dog was found dead.

Body weights:

Day......	1	9	18	24	31	38	45	52	59	66	73
Kilos.....	7.1	6.9	6.6	6.3	6.2	6.0	6.0	5.9	5.8	5.6	5.8

Day..........	80	87	94	101	108	115	124	132	143
Kilos.........	5.8	5.6	5.6	5.5	5.2	5.3	5.2	5.0	5.1

Dog S.—Offered 130 gm. of Food 3 daily.

The dog did not relish the diet especially well and consumed only part of its food during the first 25 days. 26th to 29th days. Fed Yeast A (see Chart 3). 30th to 36th days. Food intake again diminished. 37th to 50th days. Fed Tomato F (see Chart 28). The food intake diminished. It was fed Extract G with some beneficial results. 74th day. Refused food and lay down. No polyneuritic symptoms were present. Its mouth became very foul smelling. 79th day. Passed bloody stools. It was nearly dead and was killed. The case resembled the dogs of Chittenden and Underhill[8] which had pellagra-like symptoms.

Body weights:

Day......	1	9	18	24	31	38	45	52	59	66	73
Kilos.....	8.4	8.2	8.0	7.7	7.8	7.5	7.4	7.5	6.8	7.1	7.2

---

[8] Chittenden, R. H., and Underhill, F. P., *Am. J. Physiol.*, 1917, xliv, 13.

Dog X.—Offered 125 gm. of Food 3 daily.

The animal ate the food well for 22 days whereupon the food intake diminished. 28th to 31st days. Fed Yeast A (see Chart 1). The food intake again diminished until days 39 to 41 when it was fed Yeast C.

Body weights:

Day	1	8	15	24	32	43
Kilos	7.4	7.7	7.6	7.2	7.1	7.1

METABOLISM STUDIES WITH DIETS DEFICIENT IN WATER-SOLUBLE (B) VITAMINE.

By WALTER G. KARR.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.*)

(Received for publication, September 17, 1920.)

It has been suggested that vitamines may exert their unique function in intermediary metabolism or in the glandular secretory processes connected with digestion in the alimentary tract. With this possibility in mind studies of nitrogen metabolism were made with animals on similar diets respectively rich in or devoid of water-soluble (B) vitamine. The general plan was to compare the metabolism of an animal recently placed on a vitamine-free diet with its metabolism at a later period on the same diet; also to compare the metabolism on a vitamine-free diet with periods in which a supply of water-soluble (B) vitamine had been added and to control the possible supplementing action of the protein of the crude vitamine-yielding substance by a further period in which a corresponding amount of purer protein was added.

Four female dogs were employed. They were kept in ordinary metabolism cages, and the urines of different days or periods were separated by catheterization. The feces were marked into periods with carmine and after addition of alcohol containing a few drops of acid they were dried on a water bath. Total nitrogen was determined either by the Kjeldahl method or Folin's macro modification for urine.

The protein of the diet was supplied by either commercial casein[1] or commercial wheat gluten,[2] both of which are known to

---

[1] The casein was purchased from Lister Brothers, New York. It was colored very light yellow, quite finely ground, and the samples generally contained about 12.7 per cent of nitrogen.

[2] The wheat gluten was purchased from the Pure Gluten Food Company, Columbus, Ohio. It was very finely ground and contained 12 to 14 per cent of nitrogen.

TABLE I

Dog G.

Day.	Weight.	Urine N.	Daily diet.	Feces.	N balance.	N utilization.
Period 1.						
	kg.	*gm.*	*gm.*		*gm.*	*per cent*
14		5.70	Casein . . . . 50 = 6.2 gm. N	59 gm. 3.2 per cent N. 1.9 gm. N.	+2.3 or +0.5 daily.	94
15	7.4	5.42	Lard. 30			
16		5.31	Sucrose . . . 30			
17		5.25	Salts. 2			
18	7.4	5.16	Bone ash . . 5			
			About 590 calories.			
Period 2.						
28	7.2	5.60	Same as Period 1.	47 gm. 3.5 per cent N. 1.7 gm. N.	+0.2 or +0.05 daily.	93
29		5.97				
30	7.3	5.76				
31		5.60				
Period 3.						
56	7.3	6.33	Same as Period 1 except N intake = 6.32 gm.	46 gm. 3.3 per cent N. 1.5 gm N.	+0.9 or +0.2 daily.	94
57		5.36				
58	7.2	5.74				
59		6.10				
60		5.65				
(Dog develops polyneuritis, see protocol.)						
Period 4.						
77	6.9	4.17	Casein . . . 48.5 = 6.12 gm. N	90 gm. 3.5 per cent N. 3.1 gm. N.	+8.4 or +1.2 daily.	95
78		4.48				
79		4.78	Yeast.* . . 3 = 0.22 " N			
80		5.05	Lard. . . . 30 ——			
81	6.9	4.75	Sucrose . . 35 6.34 " N			
82		4.70	Salts . . . . 2 intake.			
83	6.9	4.92	Bone ash . 5			
			About 610 calories.			

---

* The brewery yeast which was supplied by the Hinckel Brewery, Albany, N. Y., was an ordinary dried commercial product containing moisture 9.1, nitrogen 7.2, and ash 6.6 per cent.

TABLE I—*Concluded.*

Day.	Weight.	Urine N.	Daily diet.	Feces.	N balance.	N utilization.
Period 5.						
	kg.	*gm.*	*gm.*		*gm.*	*per cent*
84		5.11	Casein.. 48.5 = 6.12 gm. N	71 gm.	+2.7	92
85		5.38	Gluten.. 1.5 = 0.22 " N	3.1 per	or	
86	6.9	5.27	Lard.... 30 ——	cent N.	+0.45	
87		5.55	Sucrose. 35 6.34 " N	2.1 gm.	daily.	
88		5.37	Salts.... 2 intake.	N.		
89		5.48	Bone ash 5			
			About 610 calories.			
Period 6.						
90		5.77	Casein.... 50 = 6.34 gm. N	35 gm.	+0.9	94
91		5.80	Lard...... 30	3.1 per	or	
92	6.9	5.40	Sucrose... 35	cent N.	+0.3	
			Salts..... 2	1.1 gm.	daily.	
			Bone ash . 5	N.		
			About 610 calories.			
Period 7.						
93		5.31	Casein.. 48.5 = 6.12 gm. N	59 gm.	+1.7	94
94		5.77	Yeast*.. 3 = 0.22 " N	3.1 per	or	
95		5.67	Lard.... 30 ——	cent N.	+0.3	
96	6.8	5.60	Sucrose. 35 6.34 " N	1.8 gm.	daily.	
97		5.80	intake.	N.		
			Salts.... 2			
			Bone ash 5			
			About 610 calories.			

be relatively free from water-soluble (B) vitamine. The remaining calorific requirement was furnished by lard and sucrose to which were added 2 gm. of a salt mixture and 5 gm. of bone ash, the latter to give proper consistency to the feces. Equal portions of the food were given daily soon after catheterization in the morning. Water was supplied *ad libitum.*

In Table I the data for Dog G are given in greater detail, because, as has been indicated elsewhere,[3] it is rather rare to obtain an animal which will consume, for such an extended length of time, a diet that is highly deficient in water-soluble (B) vitamine.

Protocol.—Dog G. The dog was placed on a diet which was free from the water-soluble (B) vitamine containing about 6.2 gm. of nitrogen and 620 calories. It ate the diet very eagerly. The first metabolism period was begun on the 14th day as indicated in Table I. The animal continued to receive the above mentioned diet whenever it was not on a metabolism test. After the animal was removed from the third metabolism test on the 60th day it began to vomit some of its food and on the 68th day for the first time refused to eat and showed definite symptoms of polyneuritis as previously described.[3] It ate no food again until the 72nd day, immediately after it had recovered from the polyneuritic symptoms which were alleviated by administering brewery yeast. The dog then continued to eat, consuming the original diet to which 3 gm. of yeast were added daily. Metabolism experiments were thereupon continued as indicated in the table.

DISCUSSION.

Period 3, Table I, represents the metabolic study of a dog which had been on a vitamine-free diet for 56 days and soon thereafter developed severe symptoms of polyneuritis. It had maintained its body weight and was in good nutritive condition. Apparently the intermediary metabolism of nitrogen was not materially affected by the lack of the water-soluble vitamine. Period 4 in which water-soluble (B) vitamine was supplied by brewery yeast has a larger positive nitrogen balance. The data are complicated by at least two factors: first, the supplementing action of the yeast protein; and, second, the fact that the balance experiment in this test was conducted soon after a period of decline in body weight during which the animal consumed no food (days 68 to 71). Hence the favorable result may be only the usual phenomenon of recuperation on a calorifically adequate diet. Metabolism experiments on vitamine-free diets are complicated by many factors, especially the intimate relation of water-soluble (B) vitamine to food intake,[3] and also the possible supplementing action of the protein in the crude vitamine substance used. It is obviously desirable that as soon as a good protein-free vitamine concentrate is available the above experiments be repeated using it as a source of the accessory factor.

---

[3] Karr, W. G., *J. Biol. Chem.*, 1920, xliv, 255.

TABLE II.

Period.	Days.	Body weight.	Food.*							Urine N.	Feces.			N balance.	N utilisation.
			Casein.	Gluten.	Yeast.†	Lard.	Sucrose.	Calories.	N		Total.	N			
Dog O.															
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
1	4 (10–13)‡	5.6		148		100	100	1,880	19.6	20.7	40	2.2	0.9	−2.0	95
2	4 (14–17)	5.4		144	8	100	100	1,880	19.6	19.5	41	2.2	0.9	−0.8	95
3	10 (18–27)	5.4		370		250	250	4,700	49.0	49.8	90	2.7	2.4	−3.2	94
4	5 (28–32)	5.3		180	10	125	125	2,350	24.5	21.5	59	3.3	1.9	+1.1	95
5	9 (33–41)	5.3	10	324		225	225	4,230	44.1	39.2	95	3.1	2.9	+2.0	94
6	3 (42–44)	5.3		111		75	75	1,410	14.7	13.9	34	2.8	0.9	−0.1	93
7	5 (45–49)	5.3		180	10	125	125	2,350	24.5	21.2	57	2.8	1.6	+1.7	93
Dog J.															
1	4 (15–18)	6.2		160		100	100	1,940	20.7	20.1	62	2.7	1.7	−1.1	94
2	4 (36–39)	5.5		160	8	100	100	1,960	19.6	17.3	50	2.3	1.2	+1.1	94
3	5 (46–50)	5.6		206		125	125	2,450	24.5	24.2	56	2.5	1.4	−1.1	94
4	4 (53–56)	5.6		160	8	100	100	1,950	19.6	18.1	46	2.3	1.1	+0.4	94
Dog B.															
1	5 (3–7)	10.4		240		225	225	3,900	33.6	30.3	66	2.6	1.7	+1.6	95
2	4 (10–13)	10.4	6	186		180	180	3,120	26.9	25.0	66	2.5	1.5	+0.4	94
3	5 (17–21)	10.4		233	15	225	225	3,900	33.6	31.6	59	2.8	1.7	+0.3	95

* The food was fed in equal portions each day.

† The brewery yeast which was supplied by the Hinckel Brewery, Albany, N. Y., was an ordinary dried commercial product containing moisture 9.1, nitrogen 7.2, and ash 6.6 per cent.

‡ The numbers in parentheses indicate the days of the entire period that the animal was under observation on the experimental diet. Unless indicated no vitamine was given.

The data in Table II are presented more to show the interesting supplementing action of suitable protein than the possible effect of a vitamine substance in relation to a vitamine-free diet. Small quantities of a good protein in the form of casein or yeast exerted a remarkable effect on the nitrogen balance of an animal which was consuming proteins of wheat gluten which are known not to be so "complete." In view of the small portions sufficient to produce this result, the quantities of amino-acids involved in the deficiencies must have been small at most.

The data are very consistent in showing that the nitrogen utilization in the digestive tract was unaffected by the absence of water-soluble (B) vitamine. From this we may conclude that the secretions of the glands intimately associated with digestion and absorption were not materially affected by the lack of the accessory food factors.

CONCLUSIONS.

The lack of the water-soluble accessory does not affect the utilization of the nitrogen in the alimentary tract of the dog.

It is probable that the lack of water-soluble (B) vitamine does not primarily affect the intermediary metabolism of protein nitrogen.

Data are presented which show that small amounts of a good protein beneficially supplement proteins which are known not to be so "complete."

The writer gratefully acknowledges the helpful advice of Professor Lafayette B. Mendel throughout the course of this investigation.

SOME AMINO-ACIDS FROM THE GLOBULIN OF THE COCONUT AS DETERMINED BY THE BUTYL ALCOHOL EXTRACTION METHOD OF DAKIN.

BY CARL O. JOHNS AND D. BREESE JONES.

(*From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, August 16, 1920.)

The work described in this article is chiefly confined to the determination of the amino-acids which remain in the aqueous solution of the hydrolysis products of coconut globulin after it has been subjected to the extraction with normal butyl alcohol according to the method described by Dakin (1).

By this method practically the whole of the di-basic acids and the diamino-acids are contained in the aqueous solution. Glycine and serine are extracted with difficulty, so that they may also be expected to be present in the aqueous solution.

The coconut globulin used for this hydrolysis was prepared as described in a paper previously published from this laboratory (2).

Both the diamino-acids and most of the glutaminic acid were removed before the extraction with butyl alcohol, as it was supposed that a better separation of the other products could be effected after this removal.

Satisfactory results were obtained for aspartic and glutaminic acids. The presence of hydroxyglutaminic acid could not be established. If present it must have been in very small amount. Of the amino-acids carried over by the butyl alcohol, proline was determined. A peptide anhydride was isolated and identified as leucylvaline anhydride. The question whether the origin of this dipeptide anhydride was due to incomplete hydrolysis of the protein, or whether it may have been formed as a secondary product during the extraction, is unsettled. The presence of glycine could not be established, either in the aqueous solution, or among the products that were extracted. From the aqueous solution were also obtained about 8 gm. of a mixture the properties and analysis of which indicated that it consisted largely of alanine, with an admixture of serine.

It was found necessary to continue the extraction for about 65 hours in order to arrive at the point where no more material was being carried over by the butyl alcohol. About 72 per cent of the total amount extracted was carried over, however, during the first 23 hours of the extraction.

As the products which were extracted by the butyl alcohol were accompanied by a small amount of a reddish oil, which suggested some type of decomposition, and which was difficult to remove wholly from the other products, the isolation and determination of the other amino-acids were not carried out. A separate hydrolysis of the coconut globulin and a complete determination of the hydrolysis products without the use of the butyl alcohol extraction method is published in the next paper (3). As a comparison of the methods, it can now be stated that the extraction method has given the higher yields of the di-basic acids.

The percentages of the amino-acids determined are summarized in Table I.

TABLE I.

Amino-Acids Determined.

	per cent
Glycine	0.00
Alanine	2.67
Proline	5.54
Glutaminic acid	19.07
Hydroxyglutaminic acid	0.00
Aspartic acid	5.12
Serine	1.76
Leucylvaline anhydride	0.64

EXPERIMENTAL.

459 gm. of coconut globulin, calculated on a moisture- and ash-free basis, were hydrolyzed by boiling with 1,500 cc. of 20 per cent hydrochloric acid for 27 hours. The solution was concentrated under reduced pressure to about one-half its volume, in order to remove most of the hydrochloric acid, and after diluting with an equal volume of water the suspended humin was removed by

filtration. The filtrate was largely decolorized by treatment with norite, and both the latter and the suspended humin which had been filtered off were thoroughly washed with hot water, and the washings added to the main solution. A pale yellow solution of the hydrolysis products was thus obtained. After concentrating the solution to about 1,000 cc., it was saturated, cold, with hydrochloric acid gas and allowed to stand at 0°C. for about 3 months. There were obtained in the usual way 106.22 gm. of pure glutaminic acid hydrochloride, equivalent to 85.54 gm. of the free acid, which together with a small amount obtained from a subsequent fraction amounts to 19.07 per cent of the protein. The glutaminic acid hydrochloride decomposed sharply at 197–198°C. The free acid, obtained by decomposing some of the hydrochloride with the calculated amount of potassium hydroxide, decomposed at 202°C. Determination of amino nitrogen gave the following results:

0.1380 gm. of substance gave 24.8 cc. of nitrogen (24°C., 758 mm.).

	Calculated for $C_5H_9O_4N$. *per cent*	Found. *per cent*
N.	9.52	9.52

The combined filtrates from the glutaminic acid hydrochloride were made up to 1,000 cc. For the determination of the other monoamino-acids, an aliquot was taken which represented 200 gm. of the protein. The original protein used for the hydrolysis contained 17.47 per cent of nitrogen, so the 200 gm. represented in the aliquot taken for analysis should have contained 34.95 gm. of nitrogen. However, a nitrogen determination showed that the solution contained but 34.45 gm. of nitrogen after having made corrections for the nitrogen which had been previously removed in the glutaminic acid and humin, leaving, therefore, 0.49 gm. of nitrogen unaccounted for, due to losses involved during hydrolysis, treatment with norite, and removal of glutaminic acid.

Concentrated hydrochloric acid was added to the solution to the extent of 3.5 per cent, and the basic amino-acids were removed by means of phosphotungstic acid from a volume of 2,000 cc. The bulky precipitate of phosphotungstates was washed in the usual manner and the excess of phosphotungstic acid removed with ether-amyl alcohol mixture. The hydrochloric acid was removed with silver sulfate. A determination of the total nitrogen in the solution showed that 18.81 gm. of nitrogen had been removed incident to the removal of the bases. The basic amino-acids, soluble humin, and amide nitrogen which this solution should have contained as calculated on the basis of the percentages reported in a previous publication (2) would have represented 16.61 gm. of nitrogen, leaving, therefore, 2.20 gm. unaccounted for. By thoroughly rewashing the precipitate of phosphotungstates 1.426 gm. of nitrogen were regained, still leaving 0.57 gm. unaccounted for. The combined solutions of the free amino-acids were then concentrated, and after removing 2.53 gm. of tyrosine were subjected to the butyl alcohol extraction as described by Dakin (1).

Extraction.—The solution of amino-acids was reduced to a volume of 600 cc. and extracted for about 35 hours with normal butyl alcohol according to the manner described by Dakin. The aqueous solution was then made nearly neutral with barium hydroxide and the extraction continued for about 30 hours. The extractions were run during the day, and, after standing over night, the separated amino-acids were filtered off and most of the butyl alcohol was distilled under reduced pressure, the residue being reserved for the proline determination. The extraction of the aqueous solution was resumed each morning using fresh butyl alcohol and in that way a long continued heating of any of the amino-acids in the butyl alcohol was avoided. The extraction flask containing the butyl alcohol was heated in an oil bath at a temperature adjusted so as to give a moderately rapid flow of alcohol returning to the flask. There were obtained a total of 38.52 gm. of amino-acids insoluble in the butyl alcohol. Early in the course of the extraction small amounts of a thick, reddish oil were carried over, which gradually adhered to the bottom of the flask, and could thus be largely separated from the amino-acids in suspension. During the whole extraction 4.5 gm. of this substance were obtained. It is readily soluble in water in which it reacts slightly alkaline to litmus, but is insoluble in alcohol. On long standing it showed no tendency to crystallize. Its general behavior strongly suggested it to be a product of secondary origin.

Di-Basic Acids.—The aqueous solution remaining after the extraction with butyl alcohol was concentrated to about 200 cc. and the barium hydroxide removed quantitatively with sulfuric acid. Hydrochloric acid was added to the extent of 20 to 30 per cent, and the solution boiled for 17 hours to hydrolyze any pyrrolidone carboxylic acid which may have been present. The solution was then examined in the usual way for glutaminic acid as the hydrochloride but not any was obtained. After most of the hydrochloric acid was removed by distillation under reduced pressure, the amino-acids in the solution were converted into their calcium salts according to the method of Foreman (4). 11.41 gm. of copper aspartate were obtained in the usual characteristic crystal form.

0.2033 gm. of substance (air-dried) gave 0.0591 gm. of copper oxide.

	Calculated for $C_4H_5O_4NCu.4\frac{1}{2}H_2O$. *per cent*	Found. *per cent*
Cu....................................	23.07	23.23

The filtrate from the copper aspartate, after removing the copper, was examined for hydroxyglutaminic acid, and about 9 gm. of a silver salt were obtained, the analysis of which did not agree with that of silver hydroxyglutaminate. The silver was removed with hydrogen sulfide, and on concentrating the solution 1.22 gm. of aspartic acid crystals separated. They turned red but did not melt below 280°C. Their identity was confirmed by conversion into the copper salt, the analysis and physical properties of which agreed with those of copper aspartate.

The residue obtained by evaporating the filtrate from the aspartic acid to dryness weighed 2 gm. This residue further yielded 1 gm. of glutaminic acid crystals which decomposed with effervescence at 203°C. It is therefore evident that the first separation of glutaminic acid was practically complete. The very thick syrup composing the final residue may have been hydroxyglutaminic acid, or it may have been pyrrolidone carboxylic acid derivative. The amount was so small that it was not further examined.

The alcoholic filtrate from the calcium salts, after removal of the calcium with oxalic acid, yielded 8.34 gm. of a substance having a sweetish taste. It was examined for glycine in the usual way by esterification, but no trace of glycine ester hydrochloride could be detected. The hydrochloric acid was removed with silver sulfate, and the substance recovered. Notwithstanding repeated attempts by fractional crystallization, no definite product could be obtained. The substance crystallized mostly in nodular aggregates, and at times a few plates characteristic of serine were discernible. Analysis showed the mixture to have 38 per cent of carbon and 7.26 per cent of hydrogen. Unfortunately a considerable portion of this mixture was accidentally lost. About 1 gm. of the remainder was treated with phosphotungstic acid in 10 per cent sulfuric acid, and characteristic crystals of alanine phosphotungstate were obtained. As is shown in the following publication (3), the coconut globulin contains serine, so that there is little doubt that the above mixture consisted almost wholly of alanine and serine. On this assumption the original 8.34 gm. of the mixture consisted of 5.02 gm. of alanine, and 3.32 gm. of serine, as calculated on the basis of the percentage of carbon found.

Proline.—The butyl alcoholic filtrates, containing the proline and obtained after filtering off the amino-acids, were concentrated to rather small volumes, and allowed to stand for 2 or 3 days. The substance which separated was filtered off, washed with alcohol, and the filtrate and washings were taken to dryness. The residue was then taken up with a small amount of water, the insoluble portion removed by filtration; and the filtrate again taken to dryness. By alternately extracting the residue with water and absolute alcohol in this way, there was finally obtained a product which was readily soluble both in cold absolute alcohol and water. Dakin has shown that the proline residue obtained in this way is much purer than might be expected and that "it compares favourably with the alcohol-extraction product obtained by the ester method." The total non-proline substance removed was reserved for further examination. The final proline residue was dissolved in water and made up to 500 cc. Total nitrogen and amino nitrogen determinations were made on 10 cc. aliquot portions. There were found 1.9740 gm. of total nitrogen, and 0.6248 gm. of amino nitrogen. The difference, or non-amino nitrogen, 1.3492 gm., represented 11.08 gm. of proline, equivalent to 5.54 per cent of the globulin.

Leucylvaline Anhydride.—The material which separated from the alcoholic proline solutions, previously referred to, weighed about 4 gm., of which 1.2 gm. consisted of a substance extremely difficultly soluble in water. It crystallized from absolute alcohol in a voluminous mass of long, fibrous, hair-like crystals. It was moderately soluble in boiling absolute alcohol, easily soluble in hot glacial acetic acid, difficultly soluble in dilute hydrochloric acid, and extremely difficultly soluble in boiling water. It had a bitter taste, and when heated in an open vessel sublimed in voluminous clouds, which filled the upper part of the vessel. It melted to a dark oil at 285-287°C. and gave the following results on analysis:

0.1524 gm. of substance gave 0.3477 gm. of carbon dioxide and 0.1154 gm. of water.

0.0978 gm. of substance required 9.35 cc. of 0.1 N acid.

	Calculated for $C_{11}H_{20}O_2N_2$. *per cent*	Found. *per cent*
C	62.21	62.22
H	9.43	9.73
N	13.20	13.45

All the observed properties of this compound, together with the result obtained by analysis, agree closely with those of the anhydride of the dipeptide, leucylvaline, as described by Abderhalden and Funk (5). Lack of sufficient material prevented a satisfactory identification of the products which it yielded on hydrolysis with concentrated hyrochloric acid.

The monoamino-acids which were extracted from the aqueous solution by the butyl alcohol appeared to be contaminated to a certain extent by the syrupy substance insoluble in alcohol which has been already referred to, so that the attempt to separate and determine these amino-acids was abandoned. The most soluble portions, however, were examined for glycine, both as the picrate and the ester hydrochloride, but none could be detected.

A complete determination of these amino-acids in a separate hydrolysis in now in progress.

SUMMARY.

The hydrolysis products of the globulin of the coconut were subjected to the butyl alcohol extraction method of Dakin.

Attention was confined chiefly to the amino-acids remaining in the aqueous solution after the extraction. The following values were found: glutaminic acid, 19.07; aspartic acid, 5.12; alanine, 2.67; and serine, 1.76 per cent. No hydroxyglutaminic acid or glycine could be detected.

From the amino-acids extracted by the butyl alcohol 5.54 per cent of proline, and 0.64 per cent of leucylvaline anhydride were isolated.

BIBLIOGRAPHY.

1. Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
2. Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1919, xxxvii, 149.
3. Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1920, xliv, 291.
4. Foreman, F. W., *Biochem. J.*, 1919, xiii, 378.
5. Abderhalden, E., and Funk, C., *Z. physiol. Chem.*, 1907, liii, 19.

HYDROLYSIS OF THE GLOBULIN OF THE COCONUT, COCOS NUCIFERA.

By D. BREESE JONES and CARL O. JOHNS.

(*From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, August 27, 1920.)

In the preceding paper from this laboratory (1) we have described the determination of some amino-acids from the globulin of the coconut by means of the butyl alcohol extraction method of Dakin (2). This article contains the percentages of those amino-acids which remained in the aqueous solution of the hydrolysis products after it had been subjected to extraction with butyl alcohol. These amino-acids include glutaminic and aspartic acids, serine, and alanine. The presence of neither hydroxyglutaminic acid nor glycine was established. Proline and leucylvaline anhydride, both of which were carried over by the butyl alcohol, were also determined.

Since difficulty was encountered in the separation of the other amino-acids extracted by the butyl alcohol, a new hydrolysis of the same protein has been undertaken and a complete determination of the degradation products made according to methods not involving extraction with butyl alcohol. This analysis not only gives the percentages of those amino-acids which had not been determined in the previous hydrolysis, but also furnishes supplemental data for comparison with those which had been already obtained.

Somewhat of a departure has been made from the usual order of procedure in the analysis of the hydrolysis products of proteins. Before attempting to determine any of the amino-acids, the hexone bases were first removed with phosphotungstic acid. Glutaminic acid was then removed as completely as possible as the hydrochloride, followed by removal of the remaining di-basic amino-acids. Proline and peptide anhydrides were next removed. The remaining amino-acids were then esterified, with very satisfactory results, by means of their lead salts according to the recently published method of Foreman (3). The isolation and determination of the amino-acids obtained from the esters were accomplished for the greater part in the usual way.

The results of this hydrolysis are given in Table I under Analysis 2. For comparison there is also included in this table

TABLE I.

Amino-Acids in Coconut Globulin.

Amino-acid.	Analysis 1.*	Analysis 2.	Highest val obtained.
	per cent	*per cent*	*per cent*
Glycine	0.00	Trace.	Trace.
Alanine	2.67	4.11	4.11
Valine		3.57	3.57
Leucine		5.96	5.96
Proline	5.54	2.85	5.54
Phenylalanine		2.05	2.05
Aspartic acid	5.12	3.88	5.12
Glutaminic acid	19.07	18.69	19.07
Hydroxyglutaminic acid	0.00	0.00	0.00
Serine	1.76	1.41	1.76
Tyrosine			3.18
Cystine			1.44
Arginine			15.92
Histidine			2.42
Lysine			5.80
Tryptophane			Present
Leucylvaline anhydride	0.64	0.14	0.64
Ammonia			1.57
Total			78.15

* Johns and Jones (1).

under Analysis 1, the percentages of the amino-acids obtained by means of the butyl alcohol extraction method. The last column contains the highest percentages obtained from both analyses. It is of interest to note that the summation of these figures among the highest that have been heretofore obtained in the analysis of the hydrolysis products of proteins.

The percentages of the basic amino-acids in coconut globulin as determined by the Van Slyke method, have already been published (4).

The butyl alcohol extraction method gave the higher results for aspartic and glutaminic acids, proline, and serine. The difference in the amounts of aspartic acid obtained is considerable, while the figures for glutaminic acid are in very close agreement. In neither analysis could the presence of hydroxyglutaminic acid be established.

The low result obtained for proline in Analysis 2 may be partly accounted for by the difficulty encountered when filtering and washing the excess of lime while removing the di-basic acids by the lime-alcohol method.

The percentage of alanine given under Analysis 1 shows only the amount that remained in the aqueous solution after the extraction, since that extracted by the butyl alcohol was not determined.

No glycine was obtained from Analysis 1, while its presence in small amount was detected in Analysis 2, both as the picrate and the β-naphthalenesulfone derivative.

The total amount of tyrosine isolated at various stages of the analysis amounted to 6.32 gm. or 2.3 per cent of the protein. This is within less than 1 per cent of the amount found by the colorimetric method of Folin. Several undetermined syrupy residues obtained during the analysis still gave strong positive tests for tyrosine with Millon's reagent.

All the serine which was isolated in Analysis 2 was obtained from the unesterified portion of the amino-acids. This fraction was filtered off with the barium residues which remained after liberation of the esters in chloroform with barium hydroxide.

EXPERIMENTAL.

276 gm. of the ash- and moisture-free coconut globulin were hydrolyzed by first warming on a steam bath with 1,000 cc. of hydrochloric acid (specific gravity 1.1) until practically all had dissolved. The hydrolysis was then continued by boiling in an oil bath for 36 hours. The color was largely removed from the solution by means of norite, and the bases were precipitated with phosphotungstic acid according to the method of Van Slyke. The precipitation was made from a total volume of 18 liters of solution in three separate portions of 6 liters each. After

standing for 41 hours the crystalline precipitates of phosphotungstates were removed by filtration and thoroughly washed in the prescribed manner. From the combined filtrates and washings, which had been reduced in volume to about 3 liters, there further separated on standing an appreciable amount of a crystalline phosphotungstate precipitate. In view of the fact that this second crop of phosphotungstates had separated from a solution which had been greatly reduced in volume, and which now contained about 20 per cent of hydrochloric acid, it was subjected to a thorough examination in order to eliminate the possibility of loss of any monoamino-acids which might have separated from the concentrated solution. All that could be isolated and identified consisted only of 0.23 gm. of tyrosine and 0.76 gm. of glutaminic acid. It is probable that even these had been carried down by adsorption with the phosphotungstates of the bases. Phosphotungstic acid was removed from the combined filtrates and washings from the phosphotungstate precipitates, and the solution of amino-acids concentrated to about 600 cc. After having been saturated with hydrochloric acid in the usual way, this solution gave a large yield of crystalline hydrochlorides, which were washed with cold, concentrated hydrochloric acid, dissolved in water, and reprecipitated by again saturating with hydrochloric acid gas. 50.37 gm. of glutaminic acid hydrochloride were finally obtained which decomposed with effervescence at 199–200°. By concentrating the filtrate from the reprecipitated glutaminic acid hydrochloride a second crop of crystals weighing 10.14 gm. was obtained. As this contained amino-acids other than glutaminic acid it was recrystallized from concentrated hydrochloric acid, and 3.83 gm. of glutaminic acid hydrochloride were obtained which decomposed at 199°. The traces of di-basic amino-acids remaining in the filtrate from this last crop were removed by means of the lime-alcohol method of Foreman (5) and added to the original filtrate from the glutaminic acid. From the alcoholic filtrate 1.52 gm. of phenylalanine were obtained.

The combined filtrates from the glutaminic acid hydrochloride were concentrated under reduced pressure to a thick syrup. The di-basic amino-acids still remaining in the solution were then removed as their calcium salts by means of the lime-alcohol method. Some difficulty was encountered at this stage when filtering off and washing the calcium residue which remained after adding the lime to the solution of amino-acids. The residue did not settle readily in the usual manner, but formed a sticky, gelatinous mass which filtered with great difficulty. The precipitated calcium salts yielded by the usual methods 10.70 gm. of aspartic acid, 1.50 gm. of glutaminic acid, both isolated as their copper salts, and 4.61 gm. of glutaminic acid isolated as the hydrochloride. The copper aspartate gave the following results on analysis:

0.2217 gm. of substance (air-dried) gave 0.0647 gm. of copper oxide.

	Calculated for $C_4H_5O_4NCu \cdot 4\frac{1}{2}H_2O$. *per cent*	Found. *per cent*
Cu....................................	23.07	23.32

The alcoholic filtrate from the precipitated calcium salts, amounting to about 3,500 cc., was concentrated to a syrup and diluted with water to a volume of about 1 liter. The calcium was removed by means of ammonium oxalate, and 4.28 gm. of tyrosine were isolated from the somewhat concentrated solution.

0.1571 gm. of substance gave 0.3456 gm. of carbon dioxide and 0.0888 gm. of water.

	Calculated for $C_9H_{11}O_3N$. *per cent*	Found. *per cent*
C..	59.67	60.00
H..	6.08	6.28

Chlorine was removed with silver sulfate, and the solution of amino-acids concentrated to about half its volume by distillation with a slight excess of barium hydroxide, in order to expel ammonia. The barium was removed quantitatively with sulfuric acid and the solution evaporated to dryness by distillation under reduced pressure. The residue of amino-acids thus obtained weighed 99 gm. Proline was removed in the usual way by extraction with absolute alcohol. The residue remaining after distilling off the alcohol contained 0.4 gm. of a substance which was difficultly soluble in water. This compound crystallized from absolute alcohol in long, fibrous, hair-like needles, and melted to a dark oil at 285°. It was identical in every respect with the leucylvaline anhydride previously described and isolated from this same globulin (1). The final syrupy residue of proline, which was completely soluble both in cold, absolute alcohol and in water, contained 0.4410 gm. of amino nitrogen and 1.2495 gm. of total nitrogen. The difference, or non-amino nitrogen, amounting to 0.8085 gm., is equivalent to 6.64 gm. of proline.

In order to separate the amino-acids which were insoluble in alcohol, they were esterified, and the ethyl esters fractionally distilled. The esterification was accomplished according to the method described by Foreman (3). The amino-acids were converted into their lead salts and the thoroughly dry salts suspended in about 750 cc. of absolute alcohol. The liquid was then saturated with dry hydrochloric acid gas, at first without cooling, and finally in a freezing mixture. After filtering off and washing the precipitated lead chloride, most of the free hydrochloric acid was removed by distillation at 40°, until the volume had been reduced about one-half. The hydrochloric acid still remaining was just barely neutralized with a solution of ammonia gas in absolute alcohol. After filtering off the ammonium chloride, and removing the alcohol by distillation, the residue was dissolved in dry chloroform, and the esters were liberated from their hydrochlorides by shaking with dehydrated barium hydroxide.[1] Chloroform was removed from the clear solution of esters obtained after filtering off the solid barium residue, and the esters were shaken with anhydrous ether. An appreciable amount of substance did not dissolve. Since it appeared probable that this consisted largely of ester hydrochlorides which had escaped decomposition by the barium hydroxide, it was again dissolved in chloroform and shaken with more barium hydroxide. A slow rise in temperature was noticeable. The product obtained after removing the barium residue and chloroform was now practically all soluble in ether.

The ether was removed from the united esters by distillation at atmospheric pressure and 95 gm. of esters were obtained.

The ether and chloroform, as well as the barium residues, which were removed from the esters, were reserved for further examination as follows.

---

[1] The barium hydroxide was prepared by heating the ordinary octahydrate in an open porcelain dish over a free flame until a perfectly dry residue was obtained. This was powdered and finally heated in a vacuum oven for 5 to 6 hours at 105–110°.

The Ether and Chloroform Distillates from the Esters.

The ether which had been distilled from the esters was strongly acidified with an absolute alcoholic solution of hydrochloric acid gas, and allowed to stand at 0° for about 4 weeks. There was no separation of glycine ester hydrochloride. The ether was distilled off, and the residue added to that obtained in a similar way from the chloroform distillate. The united residues were boiled with water, hydrochloric acid was quantitatively removed, and the solution evaporated to dryness. The residue, weighing 4.25 gm., consisted of a mixture of alanine, valine, and leucine. This was added to the amino-acids of Fraction I of the distilled esters.

The Barium Residues Obtained after Liberation of the Esters.

The barium residues were warmed with a slight excess of dilute sulfuric acid, and the rather large precipitate of barium sulfate was filtered off by suction and thoroughly washed. Hydrochloric acid was removed with silver sulfate, and the sulfuric acid with barium hydroxide. The solution was concentrated to about 60 cc., from which on standing 0.47 gm. of tyrosine separated. The filtrate from the tyrosine on slow evaporation yielded 3.33 gm. of serine, which separated in hard, lustrous rosettes of thick plates, or prisms. The serine, which had a somewhat sweetish taste, began to turn brown at about 210°, and at 235° (uncorrected) decomposed with effervescence to a reddish liquid. It gave the following results on analysis:

0.1731 gm. of substance gave 0.2169 gm. of carbon dioxide and 0.1013 gm. of water.

	Calculated for $C_3H_7O_3N$. *per cent*	Found. *per cent*
C	34.29	34.17
H	6.67	6.50

From the filtrate, 0.56 gm. more of serine was obtained. The final, thick, syrupy filtrate was treated with β-naphthalenesulfonyl chloride according to the method of Embdem and Tachau (6), with the idea of isolating any serine that might still remain in the filtrate. There were obtained 1.44 gm. of a product which separated from absolute alcohol in the form of hard, granular crystals. It melted to an oil at 148° (uncorrected), and gave the following results on analysis:

0.1690 gm. of substance gave 0.3341 gm. of carbon dioxide and 0.0636 gm. of water.

	Found.
	per cent
C	53.92
H	4.18

The product was readily soluble in absolute alcohol, fro which it separated again only with difficulty. In boiling wat it dissolved sparingly, and separated again on cooling, at first a fine, oily suspension which gradually crystallized forming microscopic clusters of radiating long plates or prisms. The substance now sintered at about 150°, and melted indefinitely between 160–180°. The β-naphthalenesulfone derivative of serine contains 52.88 per cent of carbon, and melts at 220° (6) while the corresponding derivative of glycine, which contains 54.34 per cent of carbon, sinters, according to Fischer and Bergell (7), at 151° and melts at 156°. It will be seen that the composition and melting point of the product obtained lie between those given for the glycine and serine derivatives, indicating a mixture of the two, consisting chiefly of the former.

TABLE II.

Fraction No.	Temperature of the bath up to	Temperature of the vapors up to	Pressure.	Weigh
	°C.	*°C.*	*mm.*	*gm.*
I	100	55	21	31
II	108	95	2	29
Distillation residue				21
Contents of the liquid air tube				2

The esters obtained after distilling off the ether were separated into fractions by fractional distillation under reduced pressure as given in Table II.

Fraction I.—The esters of this fraction were hydrolyzed by boiling with water, and 18.16 gm. of amino-acids obtained. To these were added the amino-acids obtained from the ether and chloroform distillates from the esters. By direct fractional crystallization, 11.34 gm. of alanine were isolated.

0.1743 gm. of substance gave 0.2572 gm. of carbon dioxide and 0.1207 gm. of water.

	Calculated for $C_3H_7O_2N$. *per cent*	Found. *per cent*
C..	40.41	40.24
H..	7.92	7.75

The less soluble portion of this fraction consisted of a mixture of valine and leucine. They were separated by means of the lead salt method of Levene and Van Slyke (8). 5.23 gm. of leucine were obtained as the lead salt.

0.3346 gm. of substance gave 0.2173 gm. of lead sulfate.

	Calculated for $(C_6H_{12}O_2N_2)_2Pb$. *per cent*	Found. *per cent*
Pb..	44.29	44.35

The filtrate from the lead salt of leucine yielded 6.45 gm. of valine. Analysis showed it to have the following composition:

0.1504 gm. of substance gave 0.2841 gm. of carbon dioxide and 0.1258 gm. of water.

	Calculated for $C_5H_{11}O_2N$. *per cent*	Found. *per cent*
C..	51.28	51.52
H..	9.47	9.31

The residual filtrate from the alanine, which consisted of only about 1 gm. of a syrupy substance, yielded with picric acid 0.32 gm. of a picrate which melted with decomposition at 200°. The decomposition point of glycine picrate is given by Levene and Van Slyke (9) as 202°.

Fraction II.—The amino-acids obtained from the esters of this fraction, after hydrolysis by boiling with water, yielded by the usual methods 1.22 gm. of proline, 11.20 gm. of leucine, and 3.40 gm. of valine. The leucine was isolated as the lead salt.

0.3495 gm. of substance gave 0.2263 gm. of lead sulfate.

	Calculated for $(C_6H_{12}O_2N_2)_2Pb$. *per cent*	Found. *per cent*
Pb..	44.29	44.22

Distillation Residue.—The distillation residue was shaken with a mixture of five volumes of water and an equal volume of ether. The ether extract yielded 3.80 gm. of phenylalanine.

0.1577 gm. of substance gave 0.3765 gm. of carbon dioxide and 0.0947 gm. of water.

	Calculated for $C_9H_{11}O_2N$. *per cent*	Found. *per cent*
C	65.45	65.11
H	6.66	6.67

The aqueous solution remaining after removing the phenylalanine ester with ether was boiled for 5 hours with an excess of barium hydroxide. After quantitative removal of the barium with sulfuric acid, the solution was boiled with copper carbonate, and 3.25 gm. of a bluish slate-colored copper salt were obtained. The copper was removed from the salt with hydrogen sulfide, and 1.69 gm. of a substance were obtained which contained 60.86 per cent of carbon and 6.83 per cent of hydrogen. It crystallized partly in plates resembling phenylalanine, and gave a deep, cherry-red color with Millon's reagent. The analysis and properties of this substance indicated that it was a mixture which, based on its carbon contents, consisted of 1.34 gm. of tyrosine and 0.35 gm. of phenylalanine.

The filtrate from the above copper salt, after having been freed from copper, gave a syrup from which, on long standing, no crystalline product separated. It was boiled for 14 hours with 25 per cent hydrochloric acid to regenerate any glutaminic acid which may have been converted during the preceding operations into pyrrolidone carboxylic acid. 1.51 gm. of glutaminic acid hydrochloride were obtained, which decomposed with effervescence at 197°.

The filtrate from the glutaminic acid hydrochloride was subjected to the lime-alcohol method for isolating the di-basic acids, and the latter were examined for hydroxyglutaminic acid according to the method of Dakin (2). About 2 gm. of an unpromising looking silver salt were obtained. Its silver content did not agree with that of silver hydroxyglutaminate. In view of both its small amount and impure character its further examination was discontinued.

Tyrosine.—An amount equivalent to 1 gm. of the ash- and moisture-free protein was hydrolyzed by boiling for 12 hours with 25 per cent hydrochloric acid, and the amount of tyrosine determined colorimetrically according to the method of Folin and Denis (10, 11). The tyrosine was found to comprise 3.18 per cent of the protein.

SUMMARY.

The globulin of the coconut has been hydrolyzed, and the resulting amino-acids have been determined (Table I).

By changing the order of procedure usually followed in connection with protein hydrolysis, and by applying several rather recently described methods, 78.15 per cent of the hydrolysis products of the protein used has been identified and determined.

The order of procedure followed in the isolation and determination of the amino-acids was as follows: removal of the hexone bases with phosphotungstic acid; separation of most of the glutaminic acid as the hydrochloride; precipitation of the remaining di-basic amino-acids as their calcium salts; extraction of proline and peptide anhydrides with absolute alcohol; esterification of the remaining amino-acids by means of the lead salt method of Foreman; fractional distillation of the esters under reduced pressure; and, finally, regeneration and isolation of the amino-acids in the usual manner.

Acknowledgment is hereby made to Mr. C. E. F. Gersdorff for all the carbon and hydrogen determinations made in connection with this hydrolysis.

BIBLIOGRAPHY.

1. Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1920, xliv, 283.
2. Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
3. Foreman, F. W., *Biochem. J.*, 1919, xiii, 378.
4. Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1919, xxxvii, 149.
5. Foreman, F. W., *Biochem. J.*, 1914, viii, 463.
6. Embden, G., and Tachau, H., *Biochem. Z.*, 1910, xxviii, 230.
7. Fischer, E., and Bergell, P., *Ber. chem. Ges.*, 1902, xxxv, 3779.
8. Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1909, vi, 391.
9. Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 294.
10. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 245.
11. Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1918, xxxvi, 319.

SOME PROTEINS FROM THE MUNG BEAN, PHASEOLUS AUREUS ROXBURGH.

By CARL O. JOHNS and HENRY C. WATERMAN.

(*From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, September 8, 1920.)

The mung bean, or green gram, is cultivated throughout the southern half of Asia, in the Malay Islands, and quite generally throughout the eastern portions of Africa. It is an important crop in India. It has also been cultivated with some success in various places in the middle and southern United States. The earliest varieties mature in about 80 days.

In India the seeds are used almost exclusively as human food, the straw being fed to cattle. In this country the plant has been grown both as a forage crop and as a green manure for wheat.

The beans used in this investigation were furnished by the Bureau of Plant Industry and were of the common, olive-green variety, ovoid to globose in shape, and about $\frac{3}{8}$ inch in length. The variety described by Roxburgh was the less common yellow seeded sort, which accounts for the discrepancy between the botanical name and the color of the ordinary mung bean.

The mung bean contains about 21.74 per cent of protein (N × 6.25). A series of experiments made with aqueous solutions of sodium chloride (Table I) indicated 5 per cent as the most effective concentration of this salt for the extraction. From the finely ground seed twenty volumes of 5 per cent sodium chloride solution dissolved about 19 per cent of protein, calculated on the dry weight of the meal used, or 87.5 per cent of the total protein.

The coagulation points of the proteins in the 5 per cent sodium chloride extract and their precipitation limits with ammonium sulfate were next determined. A very low coagulation point (about 40°C.), together with two others at higher temperatures, indicated an albumin and two globulin fractions. The experiments with ammonium sulfate confirmed the indication of the coagulation points regarding the globulins. Two principal fractions were precipitated, the first at from 0.15 to 0.20 of saturation with the precipitant, the second at 0.65.

On the basis of the information thus obtained the separation of the three protein fractions was undertaken. The procedure in the fractionation of the globulins was essentially the same as that used by us in isolating the globulins of the Georgia velvet bean;[1] that is, a middle fraction, from the apparent upper limit of the first main precipitate to the apparent lower limit of the second, was removed before the latter was precipitated. The middle fraction was found to be a mixture of the two globulins. That two principal globulins may thus be obtained in a considerable degree of purity is demonstrated by the fact that, in the investigation referred to, a globulin containing no detectable trace of tryptophane was separated from one which gave a strongly positive test for this amino-acid.

The albumin of the mung bean was obtained by coagulation. Its solutions were freed from soluble inorganic salts by dialysis, whereby the globulins were precipitated. The filtered dialysis liquors yielded the albumin on very slightly acidifying and heating for a long time at about 45°C.

The three types of protein preparations differ markedly in physical properties, in elementary composition, and, in the case of the two globulins, in their content of the basic amino-acids. The yield of the albumin (0.05 per cent) was so small that a determination of the basic amino-acids was impracticable.

The first globulin fraction, precipitated at 0.20 of saturation with ammonium sulfate, coagulated from its slightly acidified solutions at 95–100°C., and contained about 1.5 per cent of sulfur. It has been designated in this paper as the α-globulin. The second fraction, produced by raising the concentration of ammonium sulfate in the filtrate from the α-globulin to 0.60 of saturation, proved to be a mixture of the α- and β-globulins. The third fraction, or β-globulin, was precipitated by making the filtrate from the second fraction 0.65 saturated with the precipitant. It coagulated at 68–71°C., and contained about 0.4 per cent of sulfur. Albumin was found in the precipitates of the α-globulin and of the second fraction, and was recovered from the supernatant liquid after the precipitation by dialysis of the redissolved globulin.

---

[1] Johns, C. O., and Waterman, H. C., *J. Biol. Chem.*, 1920, xlii, 59.

The percentages of the basic amino-acids in the two globulins were determined by Van Slyke's method[2] with results as given in Table I.

TABLE I.

Basic Amino-Acids in the Mung Bean Globulins.

Amino-acid.	α-Globulin.		β-Globulin.		
	I	II	I	II	III
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cystine	1.44	1.53	0.00	0.00	0.00
Arginine	5.14	5.11	7.54	7.53	7.62
Histidine	3.26	3.35	1.97	2.04	2.04
Lysine	6.07	6.09	9.07	8.96	9.84

The method of isolation in detail, and the analysis and physical properties of the proteins are given in the following section.

EXPERIMENTAL.

Preliminary.

Extraction Experiments.—The beans were ground in a power-driven pulverizing mill. Extraction experiments were made upon the resulting fine meal with sodium chloride in various concentrations to ascertain the solvent best suited for the globulins. Samples of 10 gm. each of the meal were added to 200 gm. portions of the solvent and stirred until thorough mixture was obtained. The meal was left in contact with the solvent for 3 hours, with frequent stirring in order to secure as complete an extraction as possible. The extracts were then filtered through small folded filters and duplicate 10 cc. aliquots from each filtrate analyzed for nitrogen[3] (Table II). The maximum extraction was obtained with 5 per cent salt solution; 19.00 per cent of protein (N × 6.25), calculated on the basis of the dry meal sample, was dissolved. This solvent was selected, therefore, for the extraction of the globulin preparations.

---

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

[3] Acknowledgment is due to Mr. S. Phillips of this Laboratory for assistance in the determinations of nitrogen made in the preparation of this paper.

TABLE II.

Preliminary Extraction Experiments.

Concentration of NaCl in solvent.	Nitrogen extracted.*	Protein extracted* (N × 6.25).
per cent	*per cent*	*per cent*
0.1	2.12	13.25
1.0	2.29	14.31
3.0	2.58	16.13
5.0	3.04	19.00
7.0	2.93	18.31
9.0	2.92	18.25
11.0	2.86	17.88
4.5	2.96	18.50
5.5	2.94	18.38

* Calculated on the basis of the total dry meal used.

Coagulation Points of the Proteins in the Saline Extract.—To determine the number of proteins present in the 5 per cent sodium chloride extract and to ascertain the method best adapted for their separation, coagulation points and precipitation limits with ammonium sulfate were determined.

The apparatus used for the coagulation experiments consisted essentially of a thin walled test-tube, provided with a stirrer and thermometer, to contain the extract; the tube was suspended in a beaker which served as a water bath. A black background placed immediately behind the beaker made the slightest turbidity of the liquid in the tube clearly visible.

With a steady rise of temperature in the tube of about 0.2°C. per minute a cloud appeared at about 38°C. This increased gradually in density until the temperature reached 42.8°C., when a flocculent coagulum began to settle. After heating at this temperature for ½ hour the supernatant liquid was clear again. The very small precipitate was filtered out and the heating resumed. A second precipitate, even smaller than the first, appeared at 52–56.6°C. This was removed as before and two arge coagula were obtained at 64–75°C. and at 91–100°C. Three istinct protein fractions were thus indicated, the first of which, y reason of its small amount and very low coagulation point, as undoubtedly albumin. The large quantity and higher coagulaion points of the other two main fractions made it seem probable hat they were globulins.

Precipitation with Ammonium Sulfate.—A sample of 100 cc. f the extract was made successively from 0.05 to 0.70 saturated ith the precipitant. The dry, finely pulverized salt was used, ach portion added being entirely dissolved and the solution llowed to stand for some minutes before more was added. The irst precipitate was produced at 0.15 of saturation. On increasng the concentration of ammonium sulfate to 0.20 of saturation he precipitate was somewhat increased, became flocculent after short time, and began to settle. It was allowed to stand for ome hours, filtered, and the experiment continued with 50 cc. f the filtrate. At 0.25 of saturation no precipitate at all was roduced. At 0.30 there was but a very slight turbidity. From his point on until 0.60 of saturation was reached each addition aused very little increase in the cloudiness. At 0.60 of saturaion the still very small precipitate became flocculent and was ltered off. The filtrate, about 45 cc., was made up to 50 cc. with .60 saturated ammonium sulfate solution and another 0.05 of aturation of the solid salt added. A very heavy precipitate was nmediately formed which was not noticeably increased by bringg the concentration of the precipitant up to 0.70 of saturation. Vhen the filtrate from this fraction was saturated with ammonium ulfate only a barely visible opalescence resulted. The precipiation experiments, therefore, showed the presence of two glolins, confirming the evidence of the coagulation experiments in his regard.

On the basis of the information thus secured the isolation of the roteins was undertaken; and a number of preparations were nade. The uniformity of composition of the preparations of ach type, together with the marked difference of these types rom each other, furnished ample proof of their separate identity.

The α- and β-Globulins.

From 2 to 4 kilos of the bean meal were mixed with 5 liters per kilo of a 5 per cent aqueous solution of pure sodium chloride. The mixtures were kept in a state of vigorous agitation for 2 hours by means of a mechanical stirrer after which they were allowed to stand for from 15 to 20 hours in a cold storage chamber at a temperature of about 2°C. Enough filter paper scrap was then added to produce a mass of a consistency suitable for pressing. This pulp was placed in muslin bags and the extract expressed in a powerful press. The liquid, which contained much starch and other suspended matter, was filtered through thick mats of filter paper pulp on large Buchner funnels. The filters were washed with 5 per cent salt solution and the washings added to the filtrate.

Preparation of the α-Globulin.—Though the upper limit indicated for this fraction in the preliminary experiments was 0.20, a concentration a little higher than that necessary to precipitate the fraction resulted in a more rapid settling and easier filtration; 0.25 of saturation was used. No precipitation occurred at this point in the preliminary experiment. The precipitate became flocculent in ½ hour and settled during 15 hours standing. The supernatant, nearly clear liquid was drawn off as completely as possible by means of a siphon and passed through a folded filter previously moistened with a 0.25 saturated solution of ammonium sulfate. The precipitate was then drained on similar filters. This process was very slow, the protein acquiring a gummy consistency which made it nearly impervious to liquid. The preparation was washed from the filters with 0.25 saturated ammonium sulfate, thoroughly and repeatedly shaken with several liters of this wash solution, and again filtered off. The glutinous nature of the material was even more noticeable in this than in the first separation; and it impeded the filtration considerably. After about 24 hours, however, the wash solution was usually found to have drained off and as much as possible of the globulin was redissolved by pulping the filters in 1 or 2 liters of 5 per cent salt solution. The ammonium sulfate remaining in the precipitate was not found in this case to be sufficient to furnish a solvent for the globulin when water alone was used. The pulp from the disintegrated filters was collected on a circle of filter cloth on a small Buchner funnel and formed into a thick mat through which the globulin solution was passed twice. Considerable denaturation evidently took place during the preceding processes, for a very fine suspension, quite insoluble in neutral solvents, was found mixed with the redissolved globulin and passed repeatedly through thick pulp filter mats. The globulin was freed from mineral salts and precipitated by dialysis in parchment paper bags against running water at a temperature of 10–12°C. When the supernatant liquid in the dialyzing bags no longer gave a test for sulfates or chlorides the globulin was separated from it by decantation and washed several times with distilled water. The albumin, a part of which was thrown down from the extract with the α-globulin and remained in solution when the globulin was separated by dialysis, was thus removed. The material was finally suspended for 24 hours in absolute alcohol, filtered by suction, washed with absolute alcohol and with ether until the alcohol was washed out, and dried by heating at 110°C. in a vacuum oven.

Preparation of the β-Globulin.—The filtrate and washings from the first precipitation of the α-globulin were measured and made 0.60 saturated with ammonium sulfate. The small precipitate was filtered off, washed, redissolved, and dialyzed. A portion of the dialysis precipitate was dissolved in 5 per cent sodium chloride solution, when a determination of coagulation points showed it to be a mixture of the α- and β-globulins. The residual liquid from the dialysis of this fraction contained albumin. The filtrate from this mixed fraction was made up to 0.70 of saturation when the β-globulin separated at once as a very voluminous, flocculent mass which gave to the whole liquid the appearance and consistency of thick cream. No protein could be detected in the filtrate, and the precipitate was not, therefore, washed with ammonium sulfate solution as was the α-globulin; otherwise the procedure was the same. The yield of the β-globulin was from 5.75 to 6.00 per cent of the dry weight of the meal extracted.

Physical Properties of the Globulins.—The precipitation limits with ammonium sulfate were determined in the preliminary experiments, and are noted under that head.

The coagulation ranges of the isolated globulins were approximately the same as those found in the preliminary examination of the mixed extract, but were considerably narrower. The α-globulin coagulated between 95–100°C., the β-globulin between 68–71°C. The determinations were made on small portions of the dialysis precipitates dissolved in 5 per cent sodium chloride solution and acidified with a few drops of 1 per cent acetic acid. The results obtained with all our preparations of the globulins agree closely with the above average figures.

Microscopic examination failed to reveal any evidence of crystalline form in either of the globulins. The α-globulin was precipitated by dialysis in particles so fine as to be hardly distinguishable under a magnification of 500 diameters, while under the same power of the microscope the β-globulin presented the appearance of coarse, vitreous spheroids.

The finished preparations are fine, dusty powders, the α-globulin a very pale gray, the β-globulin cream-white in color. They absorbed 5 to 7 per cent of moisture when exposed to the air in thin layers. After drying, both globulins were insoluble in neutral solvents, but were soluble in 0.1 N alkali.

Qualitative Examination.—The ordinary protein reactions were given by both the globulins. The color reaction of tryptophane (Hopkins and Cole) was weak though undoubtedly positive with the α-globulin preparations; the β-globulin gave a much deeper color in this test. Millon's reagent yielded with both globulins a strongly positive test for tyrosine. Molisch's test indicated but traces of carbohydrate in the β-globulin, and in the α-globulin none at all could be detected.

Elementary Composition.—The hygroscopic property of dried protein preparations makes impracticable the accurate weighing of samples dried at 110°C. All the preparations were therefore brought to equilibrium with the moisture of the air before analysis was attempted, the moisture content (loss at 110°C.) was determined, and the analyses were calculated on the moisture- and ash-free basis. In order to avoid contamination with dust during exposure, and to bring about maximum absorption as rapidly as possible, the preparations were spread in thin layers in shallow dishes and placed in a large desiccator provided with tubulatures both in the side and cover. A bulb tube filled with

TABLE III.

*α-Globulin.**

	Preparation I.			Preparation II.		
	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon	52.93	53.02	52.98	53.49	53.59	53.54
Hydrogen	6.80	6.86	6.83	6.90	6.86	6.88
Nitrogen	15.53	15.50	15.52	15.78	15.64	15.71
Sulfur	1.54		1.54	1.44		1.44
Oxygen†	23.20	23.08	23.13	22.39	22.47	22.43
Ash		1.78			1.88	
	Preparation III.			Preparation IV.		
Carbon	53.99	53.91	53.95	54.02	54.08	54.05
Hydrogen	6.78	6.82	6.80	6.87	7.03	6.95
Nitrogen	15.67	15.53	15.60	15.75	15.79	15.77
Sulfur	1.50		1.50	1.53		1.53
Oxygen†	22.06	22.24	22.15	21.73	21.61	21.67
Ash		1.25			1.92	

* Analysis calculated on the ash- and moisture-free basis.

† By difference.

TABLE IV.

*β-Globulin.**

	Preparation I.			Preparation II.		
	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon	52.82	52.88	52.85	52.82	52.81	52.82
Hydrogen	6.96	6.85	6.91	6.93	6.88	6.91
Nitrogen	16.73	16.75	16.74	16.58	16.52	16.55
Sulfur	0.41	0.38	0.40	0.41		0.41
Oxygen†	23.08	23.14	23.11	23.26	23.38	23.32
Ash		0.59			0.71	
	Preparation III.			Preparation IV.		
Carbon	52.90	52.85	52.88	52.86	52.94	52.90
Hydrogen	6.79	6.99	6.89	6.85	6.87	6.86
Nitrogen	16.77	16.88	16.83	16.76	16.84	16.80
Sulfur	0.38		0.38	0.43		0.43
Oxygen†	23.16	22.90	23.03	23.10	22.92	23.01
Ash		0.22			0.43	

* Analysis calculated on the moisture- and ash-free basis.

† By difference.

absorbent cotton was attached to the upper tubulature to filter the dust from the entering air, and a slow current was drawn through the apparatus by connecting a weak suction to the side tubulature. Under these conditions the moisture content became very nearly constant in about 48 hours. The globulins were then analyzed with the results shown in Tables III and IV.

The Basic Amino-Acids and Nitrogen Distribution.—Samples of approximately 3 gm. of the globulins were hydrolyzed by boiling 24 hours with 100 cc. of 20 per cent hydrochloric acid. The hydrolysates were analyzed by Van Slyke's[3] method, using amyl alcohol and ether[4] in the decomposition of the phosphotungstates of the basic amino-acids. The figures obtained by this method for cystine are without doubt low, since the conditions of the hydrolysis are such that a notable portion of this amino-acid must be destroyed. In the case of the β-globulin, in which no cystine at all could be found in three analyses, another factor enters; namely, the solubility of cystine phosphotungstate. This was found by Van Slyke to be equivalent to 0.0026 gm. of cystine nitrogen, 0.0222 gm. of cystine, under the prescribed conditions of the analysis. It would be possible in this case, therefore, for about 0.78 per cent of cystine to have escaped detection even if none was decomposed during the hydrolysis. The figures obtained are given in Tables V, VI, VII, and VIII.

With the exception of the extremely small proportion of cystine, the nitrogen distribution of the β-globulin of the mung bean is very similar to that of other bean globulins so far examined. The α-globulin shows some differences, however. The arginine nitrogen is decidedly low, while the lysine nitrogen is not so high as in most of the bean globulins analyzed.

---

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

TABLE V.

*Distribution of Nitrogen in the α-Globulin Determined by Van Slyke's Method.**

Sample I, moisture- and ash-free, 2.7769 gm. protein, 0.4351 gm. nitrogen.†

Sample II, moisture- and ash-free, 2.7758 gm. protein, 0.4349 gm. nitrogen.†

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0405	0.0414	9.31	9.52	9.42
Humin N adsorbed by lime	0.0112	0.0121	2.57	2.54	2.56
" N in amyl alcohol extract	0.0000	0.0000	0.00	0.00	0.00
Cystine N	0.0047	0.0050	1.08	1.15	1.12
Arginine N	0.0460	0.0457	10.57	10.51	10.54
Histidine N	0.0246	0.0252	5.65	5.79	5.72
Lysine N	0.0324	0.0325	7.45	7.47	7.46
Amino N of filtrate	0.2647	0.2664	60.84	61.25	61.05
Non-amino N of filtrate	0.0104	0.0078	2.39	1.80	2.10
Total nitrogen recovered	0.4345	0.4361	99.86	100.03	99.97

* Nitrogen figures corrected for the solubilities of the phosphotungstates of the basic amino-acids.

† Nitrogen content of the protein 15.67 per cent.

TABLE VI.

Basic Amino-Acids in the α-Globulin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.44	1.53	1.49
Arginine	5.14	5.11	5.13
Histidine	3.26	3.35	3.31
Lysine	6.07	6.09	6.08

TABLE VII.

Distribution of Nitrogen in the β-Globulin Determined by Van Slyke's Method.[*]

Sample I, moisture- and ash-free, 2.8367 gm. protein, 0.4775 gm. nitrogen.[†]
" II, " " " 2.8185 " " 0.4744 " "
" III, " " " 2.8199 " " 0.4747 " "

	I	II	I	II	Average.	III	III	Average of three analyses.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*	*gm.*	*per cent*	*per cent*
Amide N	0.0569	0.0563	11.91	11.87	11.89	0.0546	11.50	11.76
Humin N adsorbed by lime	0.0096	0.0088	2.01	1.86	1.94	0.0079	1.66	1.84
" N in amyl alcohol extract	0.0005	0.0005	0.10	0.11	0.11	0.0014	0.29	0.17
Cystine N	0.0000	0.0000	0.00	0.00	0.00	0.0000	0.00	0.00
Arginine N	0.0689	0.0684	14.43	14.42	14.43	0.0692	14.58	14.48
Histidine N	0.0152	0.0156	3.18	3.29	3.24	0.0156	3.28	3.25
Lysine N	0.0494	0.0485	10.35	10.22	10.29	0.0533	11.23	10.60
Amino N of filtrate	0.2682	0.2668	56.17	56.24	56.21	0.2624	55.27	55.89
Non-amino N of filtrate	0.0103	0.0100	2.16	2.11	2.14	0.0128	2.70	2.32
Total nitrogen recovered	0.4790	0.4749	100.31	100.12	100.22	0.4772	100.51	100.31

---

[*] Nitrogen figures corrected for the solubilities of the phosphotungstates of the basic amino-acids.

[†] Nitrogen content of the protein 16.83 per cent.

TABLE VIII.

Basic Amino-Acids in the β-Globulin.

Amino-acid.	I	II	III	Average.
	per cent	*per cent*	*per cent*	*per cent*
Cystine	0.00	0.00	0.00	0.00
Arginine	7.54	7.53	7.62	7.56
Histidine	1.97	2.04	2.04	2.02
Lysine	9.07	8.96	9.84	9.29

The Albumin.

This was obtained from two sources. (1) The meal was extracted with distilled water. This dissolved the albumin, together with a considerable quantity of globulin the solution of which was made possible by the soluble mineral salts present in the meal. The salts were removed and the globulin was precipitated by dialyzing for some days against running cold water, as was done in the preparation of the globulins; then the precipitate was removed by decantation and filtration and the dialysis was continued 10 days more in repeated changes of distilled water at a temperature of about 2°C. The very small further precipitate of globulin was separated as before. From the clear solution the albumin was coagulated by slightly acidifying with acetic acid and heating for 2 hours at 45°C. The coagulation range is 37–43.5°C. The coagulum was collected on a hardened filter, washed with warm distilled water, dehydrated, and dried exactly as were the globulins. The yield was about 0.05 per cent of the meal extracted. (2) Albumin was found in the precipitates of the α-globulin and of the middle fraction and was present in supernatant liquids remaining after these fractions had been separated out by dialysis. Such solutions were further dialyzed until no more globulin separated, and after filtering the albumin was coagulated as above described. The yield from this source was 0.02 to 0.03 per cent of the original meal.

Physical Properties.—The most important of these, namely solubility in water in the absence of soluble salts, and coagulability in the presence of a trace of acid at a very low temperature, have been noted in the preceding paragraphs; these characteristics serve to define the material as albumin. The dried substance is in all respects similar to the globulin preparations.

Elementary Composition.—The analysis was carried out in exactly the same way as were those of the globulins. The result is summarized in Table IX.

TABLE IX.

*Albumin.**

	Preparation I.		
	I	II	Average.
	per cent	*per cent*	*per cent*
Carbon	54.32		54.32
Hydrogen	6.95		6.95
Nitrogen	14.83	14.68	14.76
Sulfur	1.10		1.10
Oxygen†	22.80		22.87
Ash			1.20

* Analysis calculated on the ash- and moisture-free basis.

† By difference.

Qualitative Examination.—The albumin shows all the usual protein reactions; Hopkins and Cole's test for tryptophane gives an unusually strong color, while the tyrosine reaction of Millon is slow to develop and the color deepens only slightly during long standing.

SUMMARY.

The mung bean contains about 21.74 per cent of protein (N × 6.25). Experiments with aqueous sodium chloride in various concentrations indicated a 5 per cent solution as the most effective extractant; it dissolved 19.0 per cent of protein from the finely ground seed.

The 5 per cent sodium chloride extract yielded two globulins, designated the α- and β-globulins, by fractional precipitation with ammonium sulfate and subsequent purification of the fractions as described in the foregoing pages. The yields were 0.35 and 5.75 per cent, respectively, computed on the basis of the dry material extracted. Traces of albumin, also, were obtained from extracts from which all the globulin had been precipitated by prolonged and repeated dialysis. The albumin remained in solution during the dialysis and was separated by slightly acidifying and coagulating at 45°C. The yield of the albumin was from 0.02 to 0.05 per cent of the weight of the bean meal.

Analyses showed marked differences in the nitrogen—and still fur—content of the three proteins. The globulins were sul- further distinguished from each other by marked differences in their percentages of the basic amino-acids, determined by Van Slyke's method. The β-globulin contained so little cystine that that remaining undecomposed after hydrolysis escaped precipitation by phosphotungstic acid and could not be determined by Van Slyke's method.

CHEMISTRY OF THE WHITEFISH SPERM.

By VERNON LYNCH.*

(*From the Department of Biochemistry, University of Cincinnati, Cincinnati.*)

(Received for publication, July 27, 1920.)

Preparation.

For the following analyses about 2 kilos of the ripe testicles of the common whitefish (*Coregonus albus*) of the Great Lakes were employed. This material, which had been packed in ice, was cleaned and passed through a meat grinder, and the resulting fluid mass was filtered through cheesecloth. As shown by microscopic examination, the tails of the spermatozoa were no longer visible, their disappearance being probably the result of autolysis. After the addition of an equal volume of water to reduce the viscosity, the sperm heads were thrown down as a solid white mass by rapid centrifuging. The supernatant liquid was then drawn off, and the heads were suspended in water and again centrifuged rapidly. After four such washings, the heads are pure white and do not stick together.

This material was added to three volumes of 95 per cent alcohol, after a small portion had been reserved for the determination of the water content. The mass of sperm heads was extracted four times with boiling, redistilled alcohol, and twice with redistilled ether. These extracts were united and evaporated to dryness, but the amount of lipin material obtained was negligible and was not analyzed. In the sperm, at least, the lipins are almost entirely in the cytoplasm, and no nucleus has yet been shown to contain much fatty material.

---

* The work of which the results are embodied in the following pages was carried out in my laboratory in the University of Chicago and under my direction two or three years ago. The manuscript was unfortunately mislaid and was not found until after my discharge from the Army. There has consequently been a delay of three years in the publication of the paper. In the meantime the death of the author by accident has lost to American science one of its most talented young men. Dr. Lynch's mind was unusually keen and active, and he possessed a winning personality, marked originality, courage, and vitality. The manuscript is published in the form in which he left it, with a few minor changes and corrections.

The chemistry of the sperm head is from every point of view interesting and important. Practically only three nuclei have been quantitatively analyzed; namely, the sperm head of salmon, that of the herring, and the nuclei of the corpuscles of the blood of geese. It was to secure additional information along these lines that Dr. Lynch undertook the analysis of the sperm head of the whitefish, *Coregonus albus*. The material was kindly furnished by the Booth Fisheries Company.—*Albert P. Mathews.*

It is this composition of the nucleus which in part accounts for the fact that the microscopic appearance of the nuclei remains unchanged through the course of this preparation, but the possibility of the loss of material from the nuclei is by no means excluded.

The yield of dried heads was about 100 gm.

Physical Properties.

The heads of these spermatozoa are very heavy and are readily thrown down by centrifuging. This high specific gravity is due to the large content of nucleic acid, which contains much phosphorus, and to the unusually small amount of water which they hold. The water content of some centrifuged heads, from which as much as possible of the adhering water had been removed with filter paper, was found to be 48.7 per cent. As would be expected from such concentrated nuclei, a high index of refraction is observed.

The heads appear to be little affected by a great many strong chemical reagents. They are insoluble in water, ammonia, and solutions of ordinary salts, and are only partially attacked by dilute sulfuric, nitric, hydrochloric, and acetic acids. Dilute sodium and potassium hydroxide dissolve them slowly. Of special interest is the ease with which they are attacked by dilute phosphoric acid and acid phosphate solutions. With these comparatively weak reagents, they swell up, become sticky, agglutinate, and may dissolve. Thus their solution is effected not so readily by a strong reagent as by an adequate one. The ease with which they swell after entering the egg cytoplasm is well known. In general it was noted that substances which had a solvent action upon these dead sperm heads would also agglutinate a suspension of them.

The neutral salt of protamine and nucleic acid exhibits similar properties, but Steudel[1] found evidence of a smaller molecular aggregation in the salt of clupeine and nucleic acid than that present in the sperm head. He found that a solution of the artificial salt of protamine nucleate had a lower viscosity than the same concentration of the dissolved sperm heads and he suggested to account for this that the nucleic acid in the sperm head was polymerized. Some support for this view is given later in the paper where it is shown that 1 molecule of protamine in the sperm head has united with it 4 molecules of nucleic acid.

Ultimate Analysis.

The following determinations of carbon and hydrogen in the alcohol-ether-extracted sperm heads were made by Liebig combustions.

Sample.	CO_2	C	
gm.	*gm.*	*gm.*	*per cent*
0.3478	0.5378	0.1467	42.16
0.2456	0.3778	0.1030	41.97
0.5642	0.8671	0.2365	41.94
Average			42.02

Sample.	H_2O	H	
gm.	*gm.*	*gm.*	*per cent*
0.3478	0.1813	0.0201	5.79
0.2456	0.1309	0.0145	5.91
0.5642	0.2841	0.0316	5.60
Average			5.77

The nitrogen determinations were made by the Gunning-Arnold Kjeldahl method.

---

[1] Steudel, H., *Z. physiol. Chem.*, 1913, lxxxiii, 72.

Sample.	N	
gm.	*gm.*	*per cent*
0.4943	0.1064	21.52
0.3679	0.0784	21.31
Average....................................		21.41

The phosphorus determinations were made by wet ashing as in the Neumann method and weighed as magnesium pyrophosphate.

Sample.	$Mg_2P_2O_7$	P	
gm.	*gm.*	*gm.*	*per cent*
0.4105	0.0927	0.0258	6.28
0.5485	0.1239	0.0345	6.29

The results are given in Table I, and beneath are given certain data from analyses of other nuclei for comparison.

The values for carbon and hydrogen are lower than those found in an ordinary protein. This is due principally to the large proportion of nucleic acid in which the values are even lower. The high content of nitrogen is due to the presence of a basic protein, protamine. Where the protein is a less basic histone, the values for nitrogen are lower, as in nucleohistone for example. The high content of phosphorus shows the presence of a large amount of nucleic acid, 70 per cent or more, for, as will be seen below, practically all the phosphorus is in this combination. In nuclei where the protein is less basic, the proportion of nucleic or phosphoric acid to protein is less. The ratio of phosphorus to nitrogen, being about the same in the sperm and in a neutral salt of nucleic acid and protamine, is evidence that the sperm is, in great part, a neutral protamine nucleate.

The nucleic acid nitrogen is calculated from the phosphorus content, employing the percentage formula of Steudel, $C_{43}H_{57}N_{15}P_4O_{30}$. It will be observed that the nucleic acid nitrogen makes about half the total nitrogen. The other half is almost entirely protamine nitrogen.

The ash determination is of little significance. It consists very largely of phosphoric acid, and comparison with the phosphorus percentage shows that some of the phosphoric acid has been lost, presumably by reduction of the phosphorus. It was not possible to free the ash of carbon entirely. The only base determined in the ash was calcium.

TABLE I.

Analyses of Nuclei.

	C	H	N	P	$\frac{P}{N}$	Nucleic acid nitrogen.	Protamine nitrogen.	Ash.	Ca	Fe	Nucleic acid calculated.
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Whitefish.	42.16	5.79	21.52	6.28				6.18	0.097		
Sperm heads.	41.97 41.94	5.91 5.60	21.31	6.29				5.4			
Average.......	42.02	5.77	21.42	6.29	$\frac{1}{3.404}$	10.65	10.77	5.79	0.097		70.25
Herring* sperm.	41.20	5.75	21.06	6.07	$\frac{1}{3.470}$	9.28	11.78			0	
" † "			20.78	6.42	$\frac{1}{3.237}$	10.87	9.91				
Clupeine† nucleate.					$\frac{1}{3.211}$						
Nuclei of corpuscles.‡			17.20	3.93	$\frac{1}{4.376}$	6.65	9.55				
Nucleohistone.	43.69	5.60	16.37	5.23	$\frac{1}{3.226}$	8.85	8.02				

* Mathews, A., *Z. physiol. Chem.*, 1897, xxiii, 399.

† Steudel.[1]

‡ From blood corpuscles of birds, Ackermann, D., *Z. physiol. Chem.*, 1904–05, xliii, 299.

The absence of iron is of special interest. No iron could be detected with potassium thiocyanate in the ash obtained either by the Neumann method or in the electric muffle. No organic compounds of iron were present in this extracted nucleus. Moreover, the common text-book statement that nucleoprotein always contains iron is erroneous. It may be doubted if pure nucleoprotein ever contains iron. The sperm heads contained no potassium and only 0.097 per cent of calcium. The presence of calcium in small amounts has been observed also in the other sperm heads examined, so that it seems to be generally present. This amount, however, would correspond to less than 1 atom of Ca to 5 molecules of protamine nucleate.

The attempt was now made to isolate the nucleic acid and protamine and to determine the actual amounts of these present in the sperm heads.

In the computations which follow the formula for nucleic acid of Steudel, namely $C_{43}H_{57}N_{15}P_4O_{30}$, has been the one used, and for protamine, namely the coregonine, the formula $C_{96}H_{184}N_{54}O_{22}$. Kossel and Dakin[2] suggested for salmine the formula $C_{98}H_{136}N_{54}O_{21}$. Taylor[3] suggested for salmine, $C_{96}H_{174}N_{54}O_{12}$. The formula which has been adopted above is on the hypothesis that 1 molecule of coregonine yields 12 molecules of arginine, 3 serine, 1 valine, and 2 proline groups, and that 1 molecule of water is eliminated between each two amino-acids. Then the formula should not be that of Taylor but the one given. This would be formed by the following reactions:

12 arginine ($C_6H_{14}N_4O_2$) + 3 serine ($C_3H_7NO_3$) + 1 valine ($C_5H_{11}NO_2$) + 2 proline ($C_5H_9NO_2$) = 1 salmine ($C_{96}H_{184}N_{54}O_{22}$) + 17 H_2O

This would contain 30.90 per cent N; and, if each of the arginine groups combined with 1 molecule of sulfuric acid, the sulfate $C_{96}H_{184}N_{54}O_{22} . 12\ H_2SO_4$ should contain 20.88 per cent N. I actually found 21.06 per cent. This formula requires for arginine nitrogen 88.9 per cent and for monoamino nitrogen 11.1 per cent; whereas Kossel found in this protamine 87.3 and 9.4 per cent respectively. Coregonine is evidently very closely allied to, if not identical with, salmine. From the Chinook salmon, he obtained 86.2 and 9.4 per cent respectively, and in the Rhine salmon 87.8 arginine N and 9.2 per cent of monoamino-acid N. From salveline, the protamine of the closely allied form of lake trout, he got from the alpha-salveline, 88.9 per cent basic N, and 7.1 per cent monoamino-acid N.

---

[2] Kossel, A., and Dakin, H. D., *Z. physiol. Chem.*, 1904, xli, 414.

[3] Taylor, A. E., *J. Biol. Chem.*, 1908–09, v, 394.

Furthermore since the sulfate of coregonine shows 1 molecule of sulfuric acid combined with each arginine group, making two acid equivalents to each such group, or 24 acid equivalents in all, the formula adopted for the coregonine nucleate of the sperm head is $C_{96}H_{184}N_{54}O_{22}$ $(C_{43}H_{57}N_{15}P_4O_{30})_4$ – $24H_2O$, or $C_{96}H_{160}N_{54}O_{22}$ $(C_{43}H_{51}N_{15}P_4O_{24})_4$. 100 gm. of sperm heads should yield according to this formula 32.33 gm. of protamine and 73.28 gm. of nucleic acid.

An attempt was made to separate the protamine and nucleic acid and determine them.

Nucleic Acid.

The usual method of isolating the nucleic acid from a tissue is by treatment with sodium hydroxide, precipitating the sodium nucleate with alcohol, and reprecipitating it in acid alcohol as the free acid. In the hands of Steudel[4] this method has yielded as much as 91 per cent of the theoretical amount of nucleic acid calculated from the phosphorus content of the herring sperm. Of the total phosphorus content as much as 95 per cent was actually isolated in the form of nucleic acid, and there is reason to believe that the rest was in this same combination.

Professor Mathews suggested that the nucleic acid might be completely removed from a solution of the sperm heads by precipitation with lead. Since the heads are dissolved in sodium hydroxide, a freshly prepared solution of basic lead acetate (specific gravity 1.25) was employed. Protamine is not precipitated by basic lead acetate.

In order to test the completeness of the separation, a solution of sodium nucleate was precipitated with basic lead acetate, and it was shown by examination of the filtrate that all the phosphorus had been removed. The lead salt was converted into the sodium salt with a final loss of less than 2 per cent.

A sample of the sperm heads weighing 23.33 gm. was shaken for several hours with 1,400 cc. of 1.5 per cent NaOH, and a few resistant particles were dissolved separately with a stronger solution. The nucleic acid was precipitated with an excess of the freshly prepared basic lead acetate, and the precipitate, containing lead nucleate and impurities, was washed until practically free from protamine, as shown by the biuret reaction, and then dried and weighed.

---

[4] Steudel, H., *Z. physiol. Chem.*, 1911, lxxiii, 471.

23.33 gm. of sperm heads yielded 59.885 gm. of lead precipitate. (There was a small loss of material in the foregoing analysis so that this figure is not so accurate as it should have been.) This precipitate was found to contain 4.56 per cent of N and 2.46 per cent of P. These low figures show that the precipitate contained a large amount of lead oxide and must have been the basic salt. The precipitate gave a very faint biuret reaction, showing that it was not entirely free from protamine. The ratio of P : N in the foregoing is 1 : 1.87, whereas nucleic acid requires 1: 1.66. This slight excess of N is due probably to the protamine impurity.

If all the P in the precipitate is nucleic acid, we calculate that the 59.885 gm. of precipitate must contain 16.39 gm. of nucleic acid, and as this amount was obtained from 23.33 gm. of sperm 100 gm. of sperm would yield 70.25 gm. of nucleic acid, in place of the 73.28 gm. required. If the nucleic acid is calculated from the nitrogen content, since nucleic acid contains 15.14 per cent of N, this would be 17.65 gm. of nucleic acid from 23.33 gm. of sperm or 73.92 gm. of nucleic acid from 100 gm. of sperm, in place of the 73.28 gm. required. The agreement is therefore very good; a mean of 72.08 gm. being obtained in place of 73.28 gm. calculated.

That the precipitate is nucleic acid is shown also by the close agreement of the ratio of P: N in the precipitate after an attempt to convert it to the sodium salt. 3.2096 gm. were dissolved in sodium hydroxide, partially freed from lead by passing in H_2S for 15 minutes, neutralized with acetic acid, concentrated, and precipitated with alcohol. The precipitate weighed dry 1.1483 gm. Analysis showed that it was chiefly the neutral lead salt containing 8.69 per cent of N and 5.23 per cent of P. The ratio of P: N is, however, 1:1.66, whereas nucleic acid requires 1:1.69.

Protamine.

Some insight into the nature of the protein constituent of this nucleus may be had from the results of the protein reactions upon the extracted sperm heads:

1. Biuret. On solution in NaOH. Positive.
2. Millon. Heated. Also let stand 20 days. Negative.
3. Xanthoproteic reaction. Positive.
4. Reduced sulfur. Negative.
5. Tryptophane reactions. Adamkiewicz, glyoxylic acid, and Liebermann. Negative.
6. Ehrlich's diazobenzenesulfuric acid, on solid. Positive.

The absence of tyrosine, cystine, and tryptophane is in accord with the view that the only protein present is a protamine. The positive xanthoproteic reaction is attributed to the nucleic acid.

A quantitative estimation of the protamine was made in two ways: (1) by determining the nitrogen in the filtrate left after precipitation of the nucleic acid with lead; (2) by extracting the protamine from a sample of sperm with dilute sulfuric acid.

The filtrate left after precipitation of the nucleic acid with basic lead acetate appears to contain no other protein than protamine. It gives the characteristic strong biuret reaction and gives a precipitate with nucleic acid and alkaloidal reagents, but other protein reactions are negative and phosphorus is absent. A rough estimation of the protamine may therefore be made by determining the nitrogen in this solution, assuming that coregonine, like salmine, contains 30.93 per cent of nitrogen.

	gm.
Total N of filtrate	2.2627
Protamine, calculated	7.315

100 gm. of sperm heads would therefore have yielded 31.36 gm. of protamine. This is nearly that calculated from the above formula of 32.33 gm. In view of the fact that a small amount of protamine was still in the lead nucleinate precipitate, this must be considered as a fair agreement.

A more accurate determination of the protamine is probably presented by the sulfuric acid extraction. 9.2805 gm. of dried extracted sperm heads were extracted in a mortar with three successive portions of 10 per cent H_2SO_4 and the protamine sulfate was precipitated in five volumes of 95 per cent alcohol, allowing the precipitate to stand in the alcohol over night. The white precipitate was washed in 95 per cent alcohol and absolute alcohol and dried in a vacuum desiccator. The yield of protamine sulfate from one-half the sulfuric acid extraction fluid was 2.0851 gm., which on resolution in water and reprecipitation yielded 1.9820 gm. A nitrogen determination on this precipitate showed 21.06 per cent of N. 0.5351 gm. of protamine sulfate gave 0.1127 gm. N = 21.06 per cent. Required for $C_{96}H_{184}N_{54}O_{22}\cdot 12H_2SO_4$ is 20.88 per cent.

Thus 9.2805 gm. of sperm heads yielded 3.9640 gm. of protamine sulfate after reprecipitation, or 4.17 gm., as first precipitated. The latter figure would give 2.816 gm. of protamine. 100 gm. of sperm would at this rate have yielded 30.33 gm. of protamine, whereas the formula requires 32.33 gm.

Summarized we have then the following as the composition of the sperm chromatin.

	C	H	O	N	P	Nucleic acid.	Protamine.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Required for $C_{96}H_{160}N_{54}O_{22}(C_{43}H_{51}N_{15}P_4O_{24})_4$	42.54	4.82	24.97	21.11	6.56	73.28	32.33
Found	42.04	5.77	24.49	21.41	6.29	72.08	30.84

The agreement between these figures is so good as to leave little doubt that the head of the sperm of the whitefish is essentially a chemical compound consisting of the union of nucleic acid and protamine in the proportion of 4 molecules to 1. The union is probably not of the nature of an ammonium salt, but formed with the elimination of water as in an acid amide.

CONCLUSIONS.

1. The dried extracted sperm heads of whitefish consist of about 70 per cent nucleic acid and 30 per cent protamine, coregonine.
2. All the phosphorus, 6 per cent, is combined as nucleic acid.
3. This extracted nucleus does not contain iron.
4. The protamine is in loose combination with nucleic acid, and the nucleus is of the "dissociated" (Kossel) type. The union is probably of the amide, rather than the ammonia, type.
5. The analyses agree with the formula $C_{96}H_{160}N_{54}O_{22}(C_{43}H_{51}N_{15}P_4O_{24})_4$, and this is the probable formula of this chromatin.

THE ALKALI RESERVE OF MARINE FISH AND INVERTEBRATES.

THE EXCRETION OF CARBON DIOXIDE.

By J. B. COLLIP.

(*From the Marine Biological Station, Departure Bay, Canada.*)

(Received for publication, September 3, 1920.)

INTRODUCTION.

The results of an investigation to determine the carbon dioxide content of the blood and body fluids of such marine forms as were available for study at or in the vicinity of the Marine Biological Station, Departure Bay, Vancouver Island, British Columbia, are herein reported. As the Van Slyke-Cullen (1) apparatus furnishes a convenient and ready means of determining the carbon dioxide content and capacity of blood and thereby the alkali reserve of the same, it was used exclusively in this investigation.

Methods.

The representative forms studied were, for the most part, collected personally. Great care was taken to insure that the individual specimens, the blood or celomic fluid of which was to be examined, should be bled while in a fresh condition. As will appear more fully in a subsequent communication this is a very essential point especially as regards various molluscan types. An endeavor was made to secure specimens representative of as many of the invertebrate phyla and orders of the Pisces as was possible.

The methods employed in obtaining blood or celomic fluid from the forms studied will now be detailed.

Cœlenterata.

Two types of Medusæ and the sand anemone were examined. The results recorded for these specimens can only be taken as approximate since the methods here adopted were of a special character.

Medusæ.—Several specimens were caught in a dip net, transferred to a glass container filled with fresh sea water, and carried at once to the laboratory. They were first drained free of sea water by suspending over a wide funnel by means of a double fold of cheese-cloth. They were then gently macerated and passed through the cheese-cloth. The jelly-like mass was collected in a clean, dry test-tube. This was shaken vigorously for 3 minutes, air being admitted on several occasions. 1 cc. of the fluid was then transferred to the Van Slyke-Cullen apparatus and the analysis made in the usual manner. The evolved carbon dioxide was absorbed by the use of 10 per cent sodium hydroxide. The volume of gas was reduced to cubic centimeters of carbon dioxide at 0°C. and 760 mm. pressure held by 100 cc. of fluid.

Owing to the difficulties in the way of collecting blood or celomic fluid from marine forms without loss of carbon dioxide it was thought best to examine all specimens under uniform conditions. The various samples were therefore shaken for 3 minutes in the test-tube or 25 cc. Luer syringe with atmospheric air which was renewed on several occasions. The samples thus equilibrated with atmospheric air were submitted at once to analysis. In many instances concurrent samples were also equilibrated with alveolar air of the normal human subject after the manner suggested by Van Slyke and Cullen (2). Such specimens were then transferred by means of an Ostwald pipette to the apparatus for analysis without appreciable loss of carbon dioxide.

Sea Anemones.—The specimen was dug from a sand flat at low water and carried to the laboratory in a container filled with fresh sea water. The animal was then allowed to contract in a dry open vessel. A slit was next made in the side by means of a sharp scalpel and the cavity drained free of the fluid. The soft parts were macerated in a mortar and finally suspended in an equal volume of fresh distilled water. 2 cc. of the suspension were analyzed and the results interpreted as being approximately indicative of the carbon dioxide content of the tissues of the animal.

Brachiopoda.

The specimens were collected at low water from the rocks closely adjacent to the laboratory and examined immediately. It was found that from 1 to 2 cc. of fluid could be obtained from these small forms by aspirating into a Luer syringe from the celomic cavity through a needle passed through the soft tissue exposed at the hinge.

Echinodermata.

Starfish.—These were collected near the laboratory at low water and examined immediately. The celomic fluid was aspirated from the celomic pouch of one of the arms. This was entered dorsally and laterally.

Sea Urchins.—The celomic fluid was aspirated from a celomic pouch in much the same manner as in the case of the starfish.

Arthropoda.

Crustacea.—Several species of Crustacea were studied. Blood was obtained from the individual specimens by introducing a hypodermic needle into the pericardium and aspirating into a Luer syringe. In the case of the larger forms such as *Echidnocerus formatus* and *Cancer magister* a small hole was first trephined through the carapace just dorsal to the pericardium. The needle was then introduced through this opening. It was found that the needle could be passed directly through the carapace of small crabs and shrimps. Blood was obtained from the pericardial sinus of the eagle barnacle (*Balanus aquilla*) after a small hole had been drilled through the calcareous shell just dorsal to the sinus.

Mollusca.

Pelecypoda.—Many forms representative of different species of the pelecypod Mollusca were examined. The celomic fluid or "clam juice" was obtained in all instances by aspirating from the pericardial cavity. The needle was introduced into the sinus through the soft tissue in the mid-line dorsal to the latter.

Gastropoda.—Four different species were obtained for study. Celomic fluid was obtained from the pericardial sinus of the two shell forms *Polynices* (Lunatia) *lewisii* and *Thais lamellosa*, after a small opening had been drilled in the shell adjacent to the sinus. The fluid exuded from the foot of *Polynices lewisii* was also analyzed. A direct puncture in the dorsal region allowed the aspiration of body fluid from the nudibranch *Anisodoris.*

Amphineura.—The large form *Cryptochiton* was the only representative of this class examined. Fluid was obtained from the pericardial cavity by aspirating through a needle introduced into the latter between two adjacent valves.

Cyclostomata.

The lamprey *Entosphenus tridentatus* was obtained while ascending a creek to spawn. Blood was obtained by severing the dorsal aorta posterior to the last gill slit. The blood was oxalated as collected.

Pisces.

Blood was collected from various specimens by severing the caudal vessels. It was oxalated as obtained and examined at once.

Reptilia.

Two garter snakes were caught while feeding at the water's edge. They were bled from the caudal artery. The analysis of this blood was made for comparative purposes only.

RESULTS AND DISCUSSION.

The results of the analyses of the various samples are shown in Table I.

As might be anticipated the carbon dioxide content of the blood or celomic fluid of marine forms is relatively very low as compared with the blood of mammals. The carbon dioxide content of sea water in the region from which the specimens were secured is of a widely varying quantity. The influx of a large volume of fresh water emanating from the water-sheds of the coast ranges, the varying effects of tides, winds, and currrents all contribute towards a constantly changing density of the sea water in this vicinity. In Table II is shown the density corrected to 15°C. of sea water collected daily from the surface off the landing stage of the Marine Station, Departure Bay, for a consecutive period of 33 days, also the density of depth samples taken as indicated by means of the Nansen water bottle. There are also shown the temperature and the alkalinity of the various samples in terms of cubic centimeters of 0.01 N sodium hydroxide required just to discharge the pink color imparted to 100 cc. of sea water by phenolphthalein and the buffer value of Sörensen (3) or the reactivity of Moore and Wilson .(4). This latter factor is the amount of 0.01 N acid required to carry 100 cc. of sea water from the phenolphthalein to the methyl orange point. Moore, Prideaux, and Herdman (5) found that the reactivity of sea water taken at Port Erin did not show seasonal variation. It is evident, however, as the titration figures indicate that the buffer value of sea water in the vicinity of Departure Bay varies directly as the density. Moore and coworkers (5) state that this buffer effect is due in the main to dissolved magnesium bicarbonate and not calcium bicarbonate as usually stated. The slight variation of the alkalinity of the water from day to day is of interest. It is probably associated, as Moore, Prideaux, and Herdman (5) have pointed out, with the varying intensity of photosynthesis by microscopic organisms. Surface and depth tows made daily during the summer months by Mounce[1] showed great variation in the relative amounts, and in the distribution of diatoms. The falling off of the degree of alkalinity of sea water with increasing depth is suggestive in this connection. Sörensen (3) found that deep sea water was less alkaline than that at the surface. The total carbon dioxide content of surface samples of sea water obtained in Departure Bay during the summer of 1920 was found to vary between 2 and 4 volumes per cent, the amount varying directly with the specific gravity. It is therefore of interest to find that the amount of carbon dioxide in the blood or

---

[1] Unpublished results quoted by courtesy of Miss Irene Mounce.

TABLE I.

Specimen.	CO₂ content of 100 cc. of fluid equilibrated with atmospheric air.	CO₂ content of 100 cc. of fluid equilibrated with alveolar air.	Remarks.
Jellyfish.	*cc.*	*cc.*	
10 *Phyllidium*	4.6		Specimen drained, then passed through cheesecloth.
3 *Aurelia flavidula*	5.1		" " " " " "
Sea anemone.			
Holocampa (sand anemone)	6.7		Specimen drained, macerated, and mixed with an equal volume of distilled water. Reading × 2.
Brachiopod.			
Terebrctella transversa	6.8		Obtained at low water off rocks of Jesse Island.
Starfish.			
Pisaster ochracea	6.0		" " shore of Departure Bay.
" "	6.0		" " "
" "	5.0		" " "
" "	4.2		" " "
" "	4.1	9.8	" " "
" "	6.5		" " "
Dermasterias imbricata	5.5		" " "
" "	5.5	8.8	" " "
Evasterias troschelii	6.0		" " "
Sea urchin.			
Strongylocentrotus drobachiensis	10.2		Obtained at low water off shore of Departure Bay.
" "	10.2		" " "
" "	[illegible]		" " "
" "			

" "	27.9		" " "
" "	18.4		" " "
" *productus*	20.3		" " "
" "	23.0		" " "
" "	24.2		" " "
Echidnocerus formatus	20.0		Obtained in cod nets off Nanoose Bay.
" "	22.5	28.0	" " "
Hemigrapsus nudus	24.2		Obtained off shore of Departure Bay.
Epialtus productus	10.2		" near " " "
Upogebia pugettensis (sand shrimp)	10.2		" in sand at low water in Departure Bay.
Balanus aquilla	11.2		" off reef " False Narrows.
Mollusca.			
Schizothoerus nuttalli	8.1		Obtained at low water from sand at Protection Gap.
" "	8.3		" " " "
" "	9.9	14.4	" " " "
2 " "	9.0		" " " "
Saxidomus gigantea	8.1		Obtained at low water from the shore at Departure Bay.
" "	10.2		" " " "
6 " "	8.5	14.0	" " " "
15 " "	7.8		" " " "
Paphia staminea	27.9		" " " "
" "	27.0		" " " "
8 " "	29.0		" " " "
11 " "	12.8		" " " "

TABLE I—*Continued*.

	Specimen.	CO$_2$ content of 100 cc. of fluid equilibrated with atmospheric air.	CO$_2$ content of 100 cc. of fluid equilibrated with alveolar air.	Remarks.
		cc.	cc.	
	Mollusca—*Continued*.			
	Mya arenaria	10.2		Obtained at low water from the shore at Hammond Bay.
	" "	8.3		" " " "
14	" "	8.0	13.0	" " " "
	" "	8.3		" " " "
10	" "	6.5	13.0	Kept in running sea water several days.
12	" "	6.5		" " "
11	" "	6.5		" " "
	" "	6.5		Bled as dug at Protection Gap.
	Macoma secta	11.2		Obtained at low water from shore, Hammond Bay.
	Entodesma saxicola	9.1		Obtained at low water off reef, False Narrows.
	Penitella penita	22.8		" " " " " " " "
	Mytilus edulis	9.0		Obtained at low water from shore at Departure Bay.
	" "	8.1		" " " "
	" "	9.9		" " " "
	Modiola modiolus	11.0		Obtained at low water from Protection Gap.
	Cryptochiton	6.8		" off False Narrows Reef at low water.
	"	9.0		" from Jesse Island at low water.
	Cardium corbis	6.4		" at low water off Departure Bay.
	" "	7.9		" " " " " " "
6	" "	7.5	15.0	" " " " " " "
		8.4		" " " " " Protection Gap.

Mollusca—Concluded.			
Anisodoris nobilis	7.4		Obtained at low water off Jesse Island.
“ *troschelii*	7.0		“ “ “ “ “ “ “
Polynices (Lunatia) *lewisii*	21.5		“ “ “ “ “ Protection Gap.
“ “ “	18.1		“ “ “ “ “ Departure Bay.
“ “ “	18.6		“ “ “ “ “ “ “
“ “ “	19.5		“ “ “ “ “ “ “
“ “ “	17.5		“ “ “ “ “ “ “
“ “ “	12.5		“ “ “ “ “ Protection Gap.
“ “ “	21.0		“ “ “ “ “ Departure Bay.
Fluid from foot of above	16.5		
Polynices (Lunatia) *lewisii*	20.7		“ “ “ “ “ “ “
Fluid from foot of above	17.1		
Polynices (Lunatia) *lewisii*	17.1		“ “ “ “ “ “ “
Fluid from foot of above	17.1		
Thais lamellosa	18.5		“ “ “ “ “ Jesse Island.
Puncturella	13.5		“ “ “ “ “ reef at False Narrows.
Lamprey.			
Entosphenus tridentatus	9.4		Plasma. Obtained in mill creek, Nanaimo.
Dogfish.			
Squalus sucklii	5.6	13.5	“ “ “ nets, Nanoose Bay.
“ “	5.6		“ “ “ “ “ “
“ “	5.6		Whole blood “ “ “ “ “
“ “ (dead)	3.7		“ “ “ “ “ “ “
Shark (mud shark, weight 800 lbs.)	9.3	16.2	Plasma. Obtained in net, Nanoose Bay.

TABLE I—*Concluded.*

Specimen.	CO_2 content of 100 cc. of fluid equilibrated with atmospheric air.	CO_2 content of 100 cc. of fluid equilibrated with alveolar air.	Remarks.
Ratfish.	*cc.*	*cc.*	
3 *Hydrolagus colliei* (sluggish).......	3.7	11.2	Whole blood. Obtained in nets, Nanoose Bay.
“ “ “	4.8		Plasma. “ “ “ “ “
Teleosts.			
Enophrys bison....................	12.6		Whole blood. Obtained from Departure Bay.
“ “		24.0	“ “ “ “ “ “
“ “	12.6		“ “ “ “ “ “
“ “		19.6	“ “ “ “ “ “
“ “	11.4		“ “ “ “ “ “
Anoplarchus atropurpureus (sluggish)..........................	7.0		“ “ “ at reef, False Narrows.
Phanerodon furcatus..............	10.8		“ “ “ “ Protection Gap.
2 *Porichtys notatus*.................	10.8		“ “ “ “ “ “
Sebastodes (sluggish).............	8.3		“ “ “ from Nanoose Bay.
“	10.2		“ “ “ “ Departure Bay.
“	11.2		Plasma. “ “ “ “
3 *Clupea harengus*..................	11.2		“ “ “ “ “
Platichthys stelatus................	10.2		“ “ “ “ “
Garter snake.			
Thamnophis.........................		45.0	Whole blood. Obtained at Hammond Bay.
“	35.0		“ “ “ “ Departure Bay.

TABLE II.

Date.	Source of sample.	Temperature.	Density at 15°C.	Alkalinity.	Reactivity.
1920		°C.		cc. 0.01 N $NaOH$	cc. 0.01 N H_2SO_4
June 27	Off landing stage at station.	15.9	1.0178	1.0	17.5
“ 28	“	17.6	1.0146	1.1	15.0
“ 29	“	19.5	1.0140	1.3	13.8
“ 30	“	18.0	1.0159	1.0	15.7
July 1	“	18.2	1.0161	0.7	14.8
“ 2	“	18.0	1.0167	1.3	15.1
“ 3	“	17.6	1.0186	1.2	16.5
“ 4	“	17.6	1.0181	1.4	13.9
“ 5	“	17.3	1.0187	1.7	15.6
“ 6	“	18.0	1.0196	1.5	17.5
“ 7	“	18.8	1.0198	1.6	17.4
“ 8	“	20.5	1.0112	1.3	13.0
“ 9	“	20.5	1.0122	1.2	13.5
“ 10	“	19.5	1.0133	1.4	13.8
“ 11	“	17.9	1.0187	1.0	17.0
“ 12	“	15.1	1.0194	1.5	17.5
“ 13	“	14.7	1.0208	0.4	19.0
“ 14	“	17.2	1.0094	0.5	12.0
“ 15	“	19.7	1.0108	0.7	12.5
“ 16	“	19.9	1.0097	1.0	12.3
“ 17	“	21.0	1.0133	0.7	13.6
“ 18	“	19.9	1.0142	1.0	13.5
“ 19	“	17.0	1.0187	0.7	17.2
“ 20	“	17.3	1.0199	1.7	16.5
“ 21	“	17.1	1.0192	1.3	16.7
“ 22	“	18.5	1.0088	0.3	11.3
“ 23	“	17.4	1.0114	1.3	13.0
“ 24	“	16.1	1.0150	1.5	14.7
“ 25	“	17.0	1.0142	1.5	14.7
“ 26	“	18.2	1.0119	1.3	13.5
“ 27	“	19.3	1.0115	1.0	13.0
“ 28	“	17.8	1.0115	1.0	13.0
“ 29	“	18.7	1.0120	1.0	13.7
“ 5	Surface sample off Five Fingers.	17.2	1.0203	1.6	19.0
“ 5	At 5 fathoms off Five Fingers.	15.41	1.0215	1.5	19.0
“ 5	At 10 fathoms off Five Fingers.	10.00	1.0225	0.2	20.5
“ 5	At 20 fathoms off Five Fingers.	8.98	1.0252	0.1	20.8

TABLE II—*Concluded.*

Date.	Source of sample.	Temperature.	Density at 15°C.	Alkalinity.	Reactivity.
1920		°*C.*		*cc.* 0.01 N $NaOH$	*cc.* 0.01 N H_2SO_4
July 5	At 50 fathoms off Five Fingers.	7.92	1.0267	0.1	21.0
" 5	At 100 fathoms off Five Fingers.	8.01	1.0267	−0.1	21.0
" 12	At 1 fathom off Departure Bay.	14.3	1.0214	2.0	18.5
" 12	At 2 fathoms off Departure Bay.		1.0217	1.5	18.6
" 12	At 3 fathoms off Departure Bay.		1.0220	1.4	18.5
" 12	At 5 fathoms off Departure Bay.		1.0222	1.0	18.6
" 12	At 10 fathoms off Departure Bay.		1.0233	−0.3	21.8
" 12	At 20 fathoms off Departure Bay.		1.0255	−1.4	24.0

celomic fluid of the various marine forms investigated is invariably greater than the maximum value for total carbon dioxide in sea water. The amount of combined carbon dioxide in the body tissues of Medusæ and sea anemones, the celomic fluid of brachiopods, starfish, certain sea urchins, and a number of mollusks and the blood of the dogfish and the ratfish is relatively low, but in all instances is higher than that in sea water. Certain types of Crustacea have a relatively high carbon dioxide content, such forms as *Cancer magister*, *Cancer productus*, *Echidnocerus formatus*, and *Hemigrapsus nudus* being included in this group. The kelp crab *Epialtus productus*, the sand shrimp *Upogebia pugettensis*, and the eagle barnacle *Balanus aquilla* are on the contrary comparatively low in the fixed carbon dioxide in the celomic fluid but they are in this respect somewhat analogous with the teleosts examined.

A somewhat similar phenomenon exists in the case of the Mollusca. While the majority of the forms studied have a carbon dioxide factor falling within the range between 6.3 and 11 volumes per cent certain species such as *Paphia staminea*, and *Penitella penita* of the pelecypod type and *Polynices lewisii* and *Thais lamellosa* of the gastropod class have a fixed carbon dioxide content which in nearly all instances is considerably higher than that observed in the former group.

As pointed out earlier in the paper it is essential that the blood be obtained while the animal is perfectly fresh. For instance it was noted that specimens of *Mya arenaria* bled immediately after they had been dug gave a fixed carbon dioxide factor of 6.5 volumes per cent while other specimens carried to the laboratory in fresh sea water gave a factor of 8.3 volumes per cent for combined carbon dioxide. The fixed carbon dioxide of the mollusk tends to rise rapidly when the animal is not kept in a large volume of fresh water while the opposite effect was noted in the case of the fish examined. Exposure to air causes the carbon dioxide content to fall while in the dead fish the latter may be near to that of sea water. If a fish is allowed to remain hooked but left in the open water for some little time the carbon dioxide content of the blood falls. The fish are in their reaction to injury much like the mammals as far as the alkali reserve of the blood is concerned.

The relatively low figure for carbon dioxide in the blood of the elasmobranch *Squalus sucklii* and the holocephalan *Hydrolagus colliei* stands in sharp contrast with that for the teleostian types studied.

As the hydrogen ion concentration of sea water is in most instances lower than that obtaining in the blood of marine forms and as the bicarbonate content of the latter is much higher than that of the former it is evident that the amount of the dissolved carbon dioxide in the blood or body fluids of marine forms must be considerably greater than that occurring in sea water. The tension of carbon dioxide in the blood of marine forms must also be proportionately higher than that in sea water. This brings up an interesting point. Does a process of simple diffusion furnish an adequate explanation of the mode of elimination of carbon dioxide from the blood or body fluids of marine forms to the surrounding sea water? The maintenance of a definite acid-base balance in the blood and tissues of marine forms is no doubt quite as prime an essential as in the case of higher forms. In the mammal this is largely effected by the concerted action of the respiratory and renal apparatus. The gaseous exchange between the blood and the atmosphere takes place almost entirely in the alveolar sacks where the tensions of the gases concerned are such that a process of physical diffusions seems to furnish a sufficient explanation for the passage of the oxygen inward and of the carbon dioxide outward (6). In the case of the fish conditions are somewhat different. While the respiratory and renal apparatus here as in mammals is apparently closely associated with the regulation of the reaction of the blood yet the interchange of gases between the blood and sea water taking place as it does largely through the medium of the gill filaments is on a somewhat different basis from the exchange in the mammal at the lung surface. The tension of dissolved gases in sea water to which the gill filaments are exposed cannot differ greatly from the tensions obtaining in the enveloping medium, unless it is possible that the respiratory movements are so adjusted that the tension of gases in the water of the gill cavities is kept in equilibrium with the tension of gases in the blood, in which case a process of physical diffusion would be a sufficient explanation of the mode of gaseous exchange in the fish. That this latter condition should hold seems plausible. It may also be possible that the permeability of the gill membranes to carbon dioxide is such as to allow a steep pressure gradient to exist between the dissolved carbon dioxide in the blood on the one side, and in the sea water on the other. Further experiment only can furnish a full explanation of this phenomenon.

In the case of arthropod types, such as *Cancer magister*, *Echidnocerus formatus*, and others, the combined carbon dioxide is at a much higher concentration than it is in any of the Pisces examined. A relatively greater difference must therefore exist between the tension of carbon dioxide in the blood of these forms and sea water than in the case of the Teleostei. If one is to explain the mode of carbon dioxide excretion in these forms by a process of physical diffusion one must assume that the permeability of the gill filaments to carbon dioxide is of a very low order allowing a very steep pressure gradient to be maintained between the two sides of the medium for gaseous exchange. Certain of the Mollusca have a relatively high concentration of bicarbonate in the body fluids but it may possibly be due to the anatomical features occurring here for the carbon dioxide in the sea water which is bathing the organisms to exist at a higher tension than in the open sea.

Such a difference does not exist between the bicarbonate content of the body fluids and tissues of Echinodermata, Brachipoda, and Cœlenterata, and that of sea water as has been noted in the forms above mentioned. The existence, however, of a definite pressure gradient for carbon dioxide between the tissue and the sea water suggests that a definite mechanism exists for regulating the tension of this gas in the body fluids and tissues.

SUMMARY.

1. The carbon dioxide content of the blood and the celomic fluids of various marine forms has been determined.
2. The carbon dioxide content of the blood and celomic fluid of marine forms examined equilibrated with atmospheric air is in all instances higher than the carbon dioxide content of sea water.
3. The carbon dioxide content of certain of the Arthropoda and Mollusca is relatively very high.
4. The carbon dioxide content of the blood of marine Teleostei is approximately 10 volumes per cent.
5. The carbon dioxide content of the elasmobranch *Squalus sucklii* and the holocephalan *Hydrolagus colliei* is relatively very low.
6. The alkalinity and the reactivity of several samples of sea water have been determined.
7. Surface samples of sea water in the vicinity of Departure Bay are invariably alkaline to phenolphthalein.
8. It is held that in order to maintain the constant reaction of the blood or body fluids of marine forms the carbon dioxide tension must be considerably higher in the blood and body fluids than it is in sea water.
9. The question of carbon dioxide excretion is discussed.

In conclusion I desire to express my thanks to the Curator of the Biological Station at Departure Bay, Dr. C. MacLean Fraser, for his kind assistance in making the collection of material possible, and for aid in the identification of specimens. My thanks are also due to the Biological Board of Canada for defraying the expenses in connection with this investigation.

BIBLIOGRAPHY.

1. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
2. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
3. Sörensen, S. P. L., in Moore, Prideaux, and Herdman (5), p. 174.
4. Moore, B., and Wilson, F. P., *Biochem. J.*, 1906, i, 297.
5. Moore, B., Prideaux, E., and Herdman, G., *Tr. Biol. Soc. Liverpool*, 1915, xxix, 171.
6. Krogh, A., *Skand. Arch. Physiol.*, 1910, xxiii, 274.

STUDIES ON BLOOD FAT.

I. VARIATION OF THE BLOOD FAT CONSTITUENTS OF RABBITS UNDER NORMAL CONDITIONS.

By YAJIRO HORIUCHI.

(*From the Biochemical Laboratory, Harvard Medical School, Boston.*)

(Received for publication, July 29, 1920.)

INTRODUCTION.

Since the racial and individual constancy of blood fat in the normal organism was established by Terroine[1] and confirmed by Bloor,[2] the estimation of the fat constituents in blood has become important pathologically and physiologically. During the past few years, reports on diabetes mellitus, nephritis, chronic anemia, and other diseases have appeared and also a good deal of experimental work has been published, particularly by Bloor. Up to the present time, most of the work in this line has been done on human beings and dogs. The small amount of data on the fat in the blood of rabbits was due to the lack of suitable methods for the determination of the small amount of blood fat. The Kumagawa-Suto-Shimidzu gravimetric method,[3] which usually needs 25 cc. of blood, is not available for small animals, especially for rabbits, because of the low fat constituents. To get 25 cc. of plasma, about 40 cc. of whole blood are required, and one drawing of such an amount of blood may cause a great change in blood fats, as is seen in the latter part of this paper.

The nephelometric methods for the estimation of total fatty acids and lecithin, and colorimetric method for cholesterol, proposed and modified by Bloor, were used in the present work. In these methods, 2 to 3 cc. of blood are sufficient for at least the duplicate examination of total fatty acids, lecithin, and cholesterol; and 10 cc. of blood are enough for the complete analysis of the fat constituents in whole blood, and plasma and corpuscles separately.

---

[1] Terroine, E.-F., *J. phys. et path. gen.*, 1914, xvi, 212.

[2] Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377; xix, 1; 1915, xxii, 133, 145; 1916, xxiv, 227, 447; xxv, 577.

[3] Shimidzu, Y., *Biochem. Z.*, 1910, xxviii, 237.

In all the following experiments, the blood sample was drawn from the ear vein before feeding, between 9 and 11 o'clock in the morning, after the rabbit had been kept over one night in a cage with a bowl of water. The ear was shaved, massaged, and the vein was cut slightly with a sharp razor. The quickly dropping blood was received in a graduated centrifuge tube containing 2 drops of saturated citrate or oxalate solution. The rabbit's ear should be bent a little, to keep the wound open. After one becomes proficient in the technique, it is not difficult to draw 10 cc. of blood in from 10 to 30 minutes.

The determination of blood fat constituents was carried out by Bloor's methods, except that 15 cc. of each extract of whole blood and plasma were taken instead of 10 cc. as in the original procedure, and a different amount of standard oleic acid or phosphoric acid solution was used in order to get similar values for comparison. It is not necessary to repeat the methods for the determination of blood fat constituents which have been described by Bloor. Slight modifications of the methods were made as follows:

1. Fat values of corpuscles except in the first few cases were estimated directly instead of being calculated from the values of whole blood and plasma. Rabbit blood corpuscles were separated from the plasma after centrifugation and were washed with physiological salt solution. After the wash liquid in the centrifuge tube had been thrown away, an equal quantity of distilled water was used for hemolyzing. The hemolyzed corpuscles were extracted as in the case of the whole blood and plasma.

2. For the nephelometric and colorimetric determination of the fats, 15 cc. of whole blood extract or 10 cc. of corpuscle extract were usually compared with 5 cc. of the standard oleic acid solution (containing 2 mg. in 5 cc.), or of standard phosphoric acid solution (containing 0.15 mg. of H_3PO_4 in 5 cc.), or of standard cholesterol solution (containing 0.5 mg. in 5 cc.).

3. As plasma fat constituents were found to be very low, 15 cc. of the plasma extract could be compared with only 3 cc. each

of the standard oleic acid solution, the standard phosphoric acid solution, and the standard cholesterol solution, the latter being sometimes reduced to 2 cc.

Blood Fats of Normal Rabbits and Their Variation.

Boggs and Morris[4] analyzed normal rabbit blood serum by the Soxhlet method and obtained 0.3 to 0.5 gm. of total extract. Sakai[5] determined blood fats of six rabbits by the Kumagawa-Suto-Shimidzu method, and the Autenrieth-Funk method, their average values of fatty acids, non-saponifiable substance, and cholesterol being 0.293, 0.097, and 0.063 gm., respectively.

In my experiments, the fat constituents in blood of nineteen male rabbits were analyzed. Nine of the rabbits were previously fed carrots, the other ten sunflower seeds, during periods of from 1 day to a few weeks. The former is classified as Group A, the latter as Group B; and the results are given in Table I.

The most remarkable fact in Table I is that the values of various fat constituents in plasma of rabbits on carrot diet (Group A) and on sunflower seed diet (Group B), particularly in the former, are much less than those of the corpuscles; total fatty acids in plasma being about two-thirds, while cholesterol and lecithin are only about one-half and one-third, respectively, of those of the corpuscles.

In comparing Groups A and B, total fatty acids of the former, particularly that of the plasma, were found to be considerably less than of the latter, while there was almost no difference in lecithin and cholesterol values.

In Table II, our results on the average value of rabbit blood fat constituents are compared with those of dogs and humans (Bloor).

In Table II, the most marked fact is that cholesterol and lecithin in rabbit plasma are less than those in other animals, lecithin being one-half and cholesterol being one-third that of human plasma, while no great difference in total fatty acids is found.

---

[4] Boggs, T. R., and Morris, R. S., *J. Exp. Med.*, 1909, xi, 553.

[5] Sakai, S., *Biochem. Z.*, 1914, lxii, 387.

TABLE I.

Blood Fat Constituents of Nineteen Normal Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin (Sahli).	Plasma.	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
						Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
Group A. Nine rabbits, on carrots.																				
	1918	*gm.*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						
1	Jan. 18	1,520		Clear.	33	0.20	0.19	0.23*	0.19	0.08	0.42*	0.09	0.04	0.19*	1.05	2.38	0.55	2.11	2.00	2.21
2	Mar. 7	2,340		"	38	0.33	0.29	0.39*	0.24	0.11	0.45*	0.09	0.05	0.16*	1.38	2.67	0.87	2.67	2.20	2.81
3	" 30	1,842		"	49	0.26	0.20	0.33*	0.21	0.12	0.31*	0.09	0.04	0.14*	1.24	1.68	1.06	2.33	3.00	2.21
4	June 2	1,740		"	37	0.30	0.23	0.53	0.20	0.08	0.39	0.09	0.04	0.14	1.50	2.88	1.36	2.22	2.00	2.79
	1919																			
15	Jan. 28	1,600		"	31	0.27	0.25	0.37	0.26	0.13	0.51	0.12	0.10	0.15	1.04	1.92	0.73	2.17	1.30	3.40
16	Feb. 6	2,165	55	"	37	0.32	0.28	0.34	0.21	0.11	0.38	0.11	0.06	0.18	1.52	2.55	0.89	1.91	1.83	2.11
17	" 15	1,980	55	"	37	0.30	0.21	0.31	0.18	0.10	0.36	0.10	0.07	0.14	1.67	2.10	0.86	1.80	1.43	2.57
19	Apr. 10	1,750	55	"	41	0.27	0.17	0.22	0.22	0.12	0.39	0.12	0.08	0.20	1.23	1.42	0.56	1.83	1.50	1.95
20	" 29	1,620	60	"	30	0.28	0.17	0.55	0.19	0.08	0.42	0.10	0.07	0.16	1.47	2.13	1.31	1.90	1.14	2.63
Average						0.28	0.22	0.36	0.21	0.10	0.40	0.10	0.06	0.16	1.34	2.19	0.91	2.10	1.82	2.52

* Calculated value from whole blood and plasma.

7	July 1	2,125		"	29	0.36	0.33	0.41	0.21	0.13	0.33	0.10	0.07	0.17	1.71	2.54	1.24	2.10	1.86	1.94
8	Sept. 20	1,560		"	33	0.30	0.29	0.32	0.15	0.08	0.32	0.10	0.07	0.20	2.00	3.63	1.00	1.50	1.14	1.60
9	Oct. 22	1,945		"	30	0.37	0.37	0.44	0.18	0.09	0.35	0.10	0.07	0.18	2.06	4.11	1.26	1.80	1.29	1.94
10	Nov. 11	1,190		"	32	0.34	0.34	0.31	0.21	0.16	0.31	0.12	0.10	0.18	1.62	2.13	1.00	1.75	1.60	1.72
11	" 21	2,180		"	29	0.41	0.37	0.56	0.16	0.10	0.35	0.15	0.11	0.22	2.56	3.70	1.60	1.07	0.91	1.59
12	Dec. 22	2,630	65	"	36	0.34	0.25	0.54	0.23	0.08	0.54	0.10	0.05	0.17	1.48	3.13	1.00	2.30	1.60	3.18
13	" 31	2,330		"	38	0.32	0.26	0.50	0.25	0.09	0.47	0.10	0.06	0.14	1.28	2.89	1.06	2.50	1.50	3.36
	1919																			
14	Jan. 7	1,675	60	"	37	0.30	0.32	0.25	0.25	0.14	0.43	0.12	0.06	0.20	1.20	2.29	0.58	2.08	2.33	2.15
Average						0.34	0.30	0.42	0.21	0.11	0.38	0.11	0.07	0.18	1.70	2.93	1.13	1.92	1.53	2.22

TABLE II.

Comparison of Blood Fat Constituents of Dogs, Humans, and Rabbits.

		Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						
Dog (Bloor)*		0.52			0.33			0.17			1.58			1.90		
Human (Bloor)	Men	0.36	0.38	0.36	0.30	0.22	0.40	0.21	0.22	0.19	1.20	1.68	0.89	1.44	0.98	2.33
	Women	0.36	0.40	0.29	0.29	0.19	0.44	0.23	0.24	0.21	1.31	2.15	0.69	1.29	0.82	2.14
Rabbit (Horiuchi)	Group A	0.28	0.22	0.36	0.21	0.10	0.40	0.10	0.06	0.16	1.34	2.19	0.91	2.10	1.82	2.52
	" B	0.34	0.30	0.42	0.21	0.11	0.38	0.11	0.07	0.18	1.70	2.93	1.13	1.92	1.53	2.22

* Calculated from Dogs 10, 21, 23, and 24 in Bloor, W. R., *J. Biol. Chem.*, 1915, xxiii, 323.

TABLE III.

Variation from Average of Blood Fat Constituents of Rabbits.

Rabbit No.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
Group A. Nine carrot-fed rabbits.															
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	−29	−14	−36	−10	−20	+5	−10	−33	+19	−22	+9	−40	0	−5	−12
2	+18	+32	+8	+14	+10	+13	−10	−17	0	+3	+22	−4	+28	+20	+12
3	−7	−9	−8	0	+20	−23	−10	−33	−13	−7	−23	+16	+11	+64	−12
4	+7	+5	+47	−5	−20	−45	−10	−33	−13	+12	+32	+49	+6	+9	+11
15	−4	+14	+3	+24	+30	+28	+20	+67	−6	−22	−12	−25	+3	−13	+39
16	+14	+27	−6	0	+10	−5	+10	0	+13	+13	+16	−2	−9	0	−16
17	+7	−5	−14	−14	0	−11	0	+17	−13	+25	−4	−5	−14	−22	+2
19	−4	−23	−39	+5	+20	−5	+20	+33	+25	−8	−35	−38	−13	−18	−23
20	0	−23	+53	+10	−20	+5	0	+17	0	+10	−4	+44	−10	−38	+4
Cases showing varying per cent.															
0–30 per cent.	9	8	5	9	9	8	9	4	9	9	7	5	9	7	8
30–50 " "		1	3			1		4			2	4		1	1
Over 50 per cent.			1					1						1	

Group B. Ten rabbits fed on sunflower seeds.

5	−18	−13	−24	−14	0	−16	+9	+28	−11	+14	−19	−10	−22	−20	−10
6	+6	−23	+29	+10	−18	−8	−18	−28	−28	−14	−13	+39	+33	+18	+21
7	+6	−10	−2	0	+18	−13	−9	0	−6	−7	−13	+12	+9	+22	−13
8	−12	−3	−24	−29	−27	−16	−9	0	+11	+9	+24	−10	−22	−25	−28
9	+9	+23	+5	−14	−18	−8	−9	0	0	+13	+40	+14	−6	−16	−13
10	0	+13	−26	0	+46	−18	+9	+43	0	−11	−27	−10	−9	+5	+23
11	+21	+23	+33	−24	−9	−8	−36	+57	+22	+42	+26	+32	−44	−41	−29
12	0	−17	+29	+10	−27	+42	+9	−28	−6	−19	+7	−10	+20	+5	+43
13	+6	−13	+19	+19	−18	+24	−9	−14	−22	+36	−1	−5	+30	−2	+52
14	+12	+7	−40	+19	+27	+13	+9	−14	+11	−34	+22	−48	+9	+52	−3
Cases showing varying per cent.															
0–30 per cent.	10	10	8	10	9	9	9	8	10	7	9	7	8	8	8
30–50 " "			2		1	1	1	1		3	1	3	2	1	1
Over 50 per cent.								1						1	1

There are only a few differences as regards fat constituents of corpuscles. Accordingly, the ratio of total fatty acids to lecithin in plasma is much greater in the rabbit. As the difference is more manifest in cholesterol than in lecithin, the ratio of lecithin to cholesterol is found to be larger.

Next we will consider the variation from the average of the fat constituents of whole blood, plasma, and corpuscles of the nineteen rabbits. This is, however, as Table III shows, not very large. Most of them vary from the average only within 30 per cent, as is shown in the following summary, taken from Table III.

Variation from Average.

Variation.	Total fatty acids.			Lecithin.			Cholesterol.			$\frac{\text{Total fatty acids}}{\text{Lecithin}}$			$\frac{\text{Lecithin}}{\text{Cholesterol}}$		
	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
Group A (carrot diet).															
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0–30	100	89	56	100	100	89	100	44	100	100	78	56	100	78	89
30–50		11	33			11		44			22	44		11	11
Over 50.			11					11						11	
Group B (sunflower seed diet).															
0–30	100	100	80	100	90	90	90	80	100	70	90	70	80	80	80
30–50			20		10	10	10	10		30	10	30	20	10	10
Over 50.								10						10	10

As the ratios $\frac{\text{total fatty acids}}{\text{lecithin}}$ and $\frac{\text{lecithin}}{\text{cholesterol}}$ are about constant, their variations from the average are also small.

Daily Variations of the Individual Blood Fat Constituents of Normal Rabbits.

Six rabbits were used for this purpose, two of them being given carrots, the others sunflower seeds. The former group is classified as Group A and the latter as Group B. 10 cc. of blood were drawn from each rabbit either on consecutive days for 3 or

4 days, or at intervals of a few weeks or more. The fat values of these specimens and their averages are given in Table IV. They are similar to the values in Table I, but much closer to the average.

The variations from the average in the absolute values and the ratios $\frac{\text{total fatty acids}}{\text{lecithin}}$ and $\frac{\text{lecithin}}{\text{cholesterol}}$ of blood fat constituents either on successive days or at intervals of a few weeks and also from the 1st day's value of each rabbit fed on different diets are given in Table V. The results have been summarized for discussion.

The blood fats of two carrot-fed rabbits on successive days are within 30 per cent of variation from their average. In four rabbits fed with sunflower seeds blood fats on successive days and at few weeks' intervals vary mostly within 30 per cent from their average. For the ratios $\frac{\text{total fatty acids}}{\text{lecithin}}$ and $\frac{\text{lecithin}}{\text{cholesterol}}$, the variation from the average is also very small.

The individual variation of blood fat constituents of carrot-fed rabbits on consecutive days from the 1st day's amount is without exception within 30 per cent. Most of the blood fat constituents of rabbits fed with sunflower seeds on consecutive days and at few weeks' intervals are also within 30 per cent of variation from the 1st day's value, as well as the ratios $\frac{\text{total fatty acids}}{\text{lecithin}}$ and $\frac{\text{lecithin}}{\text{cholesterol}}$. These small variations of individual blood fat constituents from the values of the first samples are very significant for the study of fat metabolism, since the constancy of blood fat constituents of normal animals from day to day is the basis of such experimental work.

Rabbit 21, for which plasma fat constituents on the successive days increased and caused abnormally large variation, was subjected to strong anemia about 2 months preceding the experiment. The increase of plasma fat constituents and the large variation from the average and the value of the first sample in this case might be due to the abnormal condition.

TABLE IV.

Variations in Blood Fat Constituents of Six Normal Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Plasma.	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
						Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
Group A. Two rabbits on carrots.																				
	1918	*gm.*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						
1	Jan. 18	1,520		Clear.	30	0.20	0.19	0.23	0.19	0.08	0.42	0.09	0.04	0.19	1.05	2.38	0.55	2.11	2.00	2.21
	" 19			"	30	0.19	0.18	0.20	0.17	0.06	0.40	0.08	0.03	0.20	1.12	3.00	0.50	2.13	2.00	2.00
	" 20			"	27	0.21	0.21	0.22	0.17	0.06	0.37	0.07	0.04	0.15	1.24	3.50	0.59	2.43	1.50	2.47
Average						0.20	0.19	0.22	0.18	0.07	0.40	0.08	0.04	0.18	1.14	2.96	0.55	2.22	1.83	2.23
	1919																			
20	Apr. 29	1,620	60	Clear.	30	0.30	0.17	0.55	0.19	0.08	0.42	0.10	0.07	0.16	1.58	2.13	1.31	1.90	1.14	2.63
	" 30		55	"	30	0.31	0.16	0.61	0.18	0.07	0.39	0.09	0.05	0.16	1.72	2.29	1.56	2.00	1.40	2.44
	May 1		62	"	28	0.28	0.21	0.63	0.19	0.08	0.42	0.10	0.05	0.17	1.47	2.63	1.50	1.90	1.60	2.47
	" 2		51	"	22	0.29	0.21	0.64	0.18	0.09	0.43	0.09	0.05	0.16	1.61	2.33	1.50	2.00	1.80	2.69
Average						0.30	0.19	0.61	0.19	0.08	0.42	0.10	0.06	0.16	1.60	2.35	1.47	1.95	1.49	2.56

[illegible] rabbits on sunflower seeds.

	1918																			
8	Sept. 20	1,560		Clear.	33	0.30	0.29	0.32	0.15	0.08	0.32	0.10	0.07	0.20	2.00	3.63	1.00	1.50	1.14	1.60
	" 21			"	22	0.27	0.23	0.37	0.13	0.07	0.35	0.08	0.05	0.19	2.08	3.29	1.63	1.63	1.40	1.84
	" 23			"	23	0.36	0.35	0.53	0.17	0.09	0.36	0.11	0.08	0.16	2.12	3.89	1.47	1.55	1.25	2.25
	Oct. 21			"	29	0.38	0.37	0.42	0.18	0.09	0.35	0.10	0.06	0.16	2.11	4.11	1.20	1.80	1.50	2.19
	" 22			"	30	0.31	0.35	0.37	0.17	0.11	0.30	0.11	0.05	0.18	1.82	3.18	1.23	1.55	2.20	1.67
	" 23			"	25	0.32	0.28	0.39	0.17	0.11	0.41	0.11	0.08	0.16	1.88	2.55	0.95	1.55	1.38	2.56
Average						0.32	0.31	0.40	0.16	0.09	0.35	0.10	0.07	0.18	2.00	3.44	1.25	1.60	1.48	2.02
9	Oct. 22	1,945		Clear.	30	0.37	0.37	0.44	0.18	0.09	0.35	0.10	0.07	0.18	2.06	4.11	1.26	1.80	1.29	1.94
	" 23			"	22	0.38	0.37	0.49	0.13	0.07	0.37	0.10	0.07	0.16	2.92	5.29	1.32	1.30	1.00	2.31
	" 24			"	23	0.38	0.39	0.58	0.16	0.12	0.36	0.11	0.09	0.17	2.38	3.25	1.19	1.45	1.33	2.12
Average						0.38	0.38	0.50	0.16	0.09	0.36	0.10	0.08	0.17	2.45	4.22	1.26	1.52	1.21	2.12
11	Nov. 21	2,180		Clear.	29	0.41	0.37	0.56	0.16	0.10	0.35	0.15	0.11	0.22	2.56	3.70	1.60	1.07	0.91	1.59
	" 22			"	30	0.39	0.40	0.40	0.17	0.10	0.32	0.19	0.10	0.19	2.29	4.00	1.25	0.89	1.00	1.68
	" 23			"	24	0.43	0.39	0.58	0.19	0.13	0.34	0.16	0.15	0.22	2.26	3.00	1.71	1.19	0.87	1.55
Average						0.41	0.39	0.51	0.17	0.11	0.34	0.17	0.12	0.21	2.37	3.57	1.52	1.05	0.93	1.61
21	Dec. 31	2,330		Clear.	38	0.32	0.25	0.45	0.25	0.09	0.47	0.10	0.06	0.14	1.28	2.78	0.96	2.50	1.50	3.36
	1919																			
	Mar. 15	2,380	60	"	38	0.24	0.21	0.29	0.29	0.12	0.56	0.12	0.06	0.18	0.83	1.75	0.52	2.42	2.00	3.11
	Apr. 2	2,370	65	"	41	0.28	0.25	0.35	0.23	0.09	0.46	0.11	0.06	0.18	1.22	2.78	0.76	2.09	1.50	2.59
	" 3		60	"	34	0.30	0.26	0.32	0.24	0.11	0.44	0.12	0.07	0.20	1.25	2.36	0.73	2.00	1.56	2.20
	" 4		55	"	30	0.34	0.37	0.37	0.25	0.13	0.46	0.12	0.09	0.19	1.36	2.85	0.80	2.08	1.44	2.42
	" 5		52	"	34	0.36	0.39	0.35	0.27	0.13	0.47	0.13	0.10	0.18	1.33	3.00	0.74	2.08	1.30	2.61
Average						0.31	0.29	0.36	0.26	0.11	0.48	0.12	0.07	0.18	1.21	2.59	0.75	2.20	1.55	2.72

TABLE V.

Summary of Variation of Blood Fat Constituents of Rabbits from Their Average and from 1st Day's Value.

Rabbit No.		Date.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
			Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
	Group A. Two carrot-fed rabbits.																
		1918															
1	1. Variation from average, *per cent.*	Jan. 18	0	0	+5	+6	−17	+5	+13	0	+6	−8	−20	0	−5	+9	−1
		" 19	−5	−5	−9	−6	0	0	0	−25	+11	−2	+1	−9	−4	+9	−10
		" 20	+5	+11	0	−6	0	−8	−13	0	−17	+9	+18	+7	+9	−18	+11
	Cases showing varying per cent.	0–30 per cent.	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
		30–50 " "															
		Over 50 " "															
	2. Variation from 1st day's amount, *per cent.*	2nd day.	−5	−5	−13	−11	−25	−5	−11	−25	+5	+7	+26	−9	+1	0	−10
		3rd "	+5	+11	−4	−11	−25	−7	−22	0	−21	+18	+47	+7	+15	−25	+12
	Cases showing varying per cent.	0–30 per cent.	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2
		30–50 " "											1				
		Over 50 " "															
		1919															
20	1. Variation from average, *per cent.*	Apr. 29	0	−11	−10	0	0	0	0	+17	0	−1	−9	−11	−3	−23	+3
		" 30	+3	−16	0	−5	−13	−7	−10	−17	0	+8	−3	+6	+3	−6	−5
		May 1	−7	+11	+3	0	0	0	0	−17	+6	−8	+12	+2	−3	+7	−4
		" 2	−3	+11	+5	−5	+13	+2	−10	−17	0	−1	−1	+2	+3	+21	+5

	Cases showing varying per cent.	0–30 per cent.	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
		30–50 " "															
		Over 50 " "															
	2. Variation from 1st day's amount, *per cent.*	2nd day.	+3	−6	+11	−5	−13	−7	−10	−29	0	+9	+8	+19	+5	+23	−7
		3rd "	−7	+24	+15	0	0	0	0	−29	+6	−7	+23	+15	0	+40	−6
		4th "	−3	+24	+16	−5	+13	+2	−10	−29	0	+2	+9	+15	+5	+58	+2
	Cases showing varying per cent.	0–30 per cent.	3	3	3	3	3	3	3	3	3	3	3	3	3	1	3
		30–50 " "														1	
		Over 50 " "														1	

Group B. Four rabbits on sunflower seeds.

		1918															
8	1. Variation from average, *per cent.*	Sept. 20	−6	−6	−20	−6	−11	−9	0	0	−11	0	+6	−13	−6	−23	−21
		" 21	−16	−26	−8	−19	−22	0	−20	−29	+6	+4	−4	−8	+9	−5	−9
		" 23	+13	+13	+33	+6	0	+3	+10	+14	0	+6	+13	+28	−3	−16	+11
		Oct. 21	+19	+19	+5	+13	0	0	0	−14	0	+6	+19	+4	+13	+1	+8
		" 22	−3	+13	−8	+6	+22	−17	+10	−29	0	−9	−8	+7	−3	+49	−17
		" 23	0	−10	−3	+6	+22	+17	+10	+14	−11	−6	−26	−17	−3	−7	+27
	Cases showing varying per cent.	0–30 per cent.	6	6	5	6	6	6	6	6	6	6	6	6	6	5	6
		30–50 " "			1											1	
		Over 50 " "															
	2. Variation from 1st day's amount, *per cent.*	Sept. 21	−10	−21	+16	−13	−13	+9	−20	−29	−5	+4	−9	+6	+9	+23	+15
		" 23	+20	+21	+66	+13	+13	+13	−10	+14	−20	+6	+7	+47	+3	+10	+41
		Oct. 21	+27	+28	+31	+20	+13	+9	0	−14	−20	+6	+12	+20	+20	+32	+37
		" 22	+3	+21	+16	+13	+38	+6	−10	−29	0	−9	−12	+23	+3	+97	+4
		" 23	+7	−3	+22	+13	+38	+28	−10	+14	−20	−6	−30	−5	+3	+21	+60
	Cases showing varying per cent.	0–30 per cent.	5	5	3	5	3	5	5	5	5	5	5	4	5	3	2
		30–50 " "			1		2							1		1	2
		Over 50 " "			1											1	1

TABLE V—*Concluded.*

Rabbit No.		Date.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
			Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
			Group B—Concluded.														
		1918															
9	1. Variation from average, *per cent.*	Oct. 22	−3	−3	−2	+13	0	−3	0	−13	+6	−16	−3	0	+18	+7	−8
		" 23	0	−3	+9	−19	−22	+3	0	−13	−6	+19	+25	−5	−14	−17	+9
		" 24	0	+5	−4	0	+33	0	+10	+13	0	−3	−21	−6	−5	+10	0
	Cases showing varying per cent.	0–30 per cent.	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3
		30–50 " "					1										
		Over 50 " "															
	2. Variation from 1st day's amount, *per cent.*	2nd day.	+3	0	+11	−28	−22	+6	0	0	−11	+42	+29	+5	−28	−22	+19
		3rd "	+3	+5	−2	−11	+33	+3	+10	+29	−6	+15	−21	−6	−19	+3	+9
	Cases showing varying per cent.	0–30 per cent.	2	2	2	2	1	2	2	2	2	1	2	2	2	2	2
		30–50 " "					1					1					
		Over 50 " "															
11	1. Variation from average, *per cent.*	Nov. 21	0	−5	+10	−6	−9	+3	−12	−8	+5	+8	+4	+5	+2	−2	−1
		" 22	−5	+3	−22	0	−9	−6	+12	−17	−10	−3	+12	−17	−15	+8	−4
		" 23	+5	0	+14	+12	+18	0	−6	+25	+5	−5	−16	+13	+13	−6	−4
	Cases showing varying per cent.	0–30 per cent.	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
		30–50 " "															
		Over 50 " "															

	2. Variation from 1st day's amount, *per cent.*	2nd day.	−5	+8	−29	+6	0	+14	+27	−9	−14	−11	+8	−22	−17	+10	+6
		3rd "	+5	+5	+4	+19	+30	+21	+7	+36	0	−12	−19	+7	−11	−4	−3
	Cases showing varying per cent.	0–30 per cent.	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2
		30–50 " "								1							
		Over 50 " "															
21	1. Variation from average, *per cent.*	Dec. 31	+3	−14	+25	−4	−18	−2	−17	−14	−22	+6	+7	+28	+14	−3	+24
		1919															
		Mar. 15	−23	−28	−19	+12	+9	+17	0	−14	0	−31	−32	−31	+10	+29	+15
		May 2	−10	−14	−3	−12	−18	−4	−8	−14	0	+1	+7	+1	−5	−3	−6
		" 3	−3	−10	−11	−8	0	−8	0	0	+11	+3	0	−3	−9	+1	−18
		" 4	+10	+28	+3	−4	+18	−4	0	+29	+6	+12	+10	+7	−5	−7	−11
		" 5	+16	+34	−3	−4	+18	−2	+8	+43	0	+10	+16	−1	−5	−16	−4
	Cases showing varying per cent.	0–30 per cent.	6	5	6	6	6	6	6	5	6	5	5	5	6	6	6
		30–50 " "		1						1		1	1	1			
		Over 50 " "															
	2. Variation from 1st day's amount, *per cent.*	Mar. 15	−25	−16	−36	+16	+33	+17	+20	0	+29	−35	−37	−46	−3	−33	−7
		May 2	−13	0	−22	−8	0	−2	+10	0	+29	−5	0	−21	−16	0	−24
		" 3	−6	+4	−29	−4	+22	−4	+20	+17	+43	−2	−15	−24	−20	+4	−35
		" 4	+6	+48	−18	0	+44	−2	+20	+50	+36	+6	+3	−17	−17	−4	−28
		" 5	+13	+56	−22	+8	+44	0	+30	+67	+29	+4	+8	−23	−17	−13	−22
	Cases showing varying per cent.	0–30 per cent.	5	3	4	5	2	5	5	3	3	4	4	4	5	4	4
		30–50 " "		1	1		3			1	2	1	1	1		1	1
		Over 50 " "		1						1							

Summary of Table V.

Group and rabbit No.		Variation.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
			Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
1. Individual variation from average.																	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Group A.	1	0–30	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
		30–50															
		Over 50.															
	20	0–30	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
		30–50															
		Over 50.															
Group B.	8	0–30	100	100	83	100	100	100	100	100	100	100	100	100	100	83	100
		30–50			17											17	
		Over 50.															
	9	0–30	100	100	100	100	67	100	100	100	100	100	100	100	100	100	100
		30–50					33										
		Over 50.															
	11	0–30	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
		30–50															
		Over 50.															
	21	0–30	100	83	100	100	100	100	100	83	100	83	83	83	100	100	100
		30–50		17						17		17	17	17			
		Over 50.															
2. Individual variation from the 1st day's value.																	
Group A.	1	0–30	100	100	100	100	100	100	100	100	100	100	50	100	100	100	100
		30–50											50				
		Over 50.															
	20	0–30	100	100	100	100	100	100	100	100	100	100	100	100	100	33	100
		30–50														33	
		Over 50.														33	
Group B.	8	0–30	100	100	60	100	60	100	100	100	100	100	100	80	100	60	40
		30–50			20		40							20		20	40
		Over 50.			20											20	20
	9	0–30	100	100	100	100	50	100	100	100	100	50	100	100	100	100	100
		30–50					50					50					
		Over 50.															
	11	0–30	100	100	100	100	100	100	100	50	100	100	100	100	100	100	100
		30–50								50							
		Over 50.															
	21	0–30	100	60	80	100	40	100	100	60	60	80	80	80	100	80	80
		30–50		20	20		60			20	40	20	20	20		20	20
		Over 50.		20						20							

SUMMARY AND CONCLUSIONS.

The blood fat constituents of nineteen normal rabbits, of different sizes and on different diets, have been determined. In six of them, analyses were carried out every day for 3 or 4 days, and also in two of the latter at different times at intervals of a few weeks.

It is most noticeable that in rabbit blood the fat constituents of the plasma are markedly less than in the corpuscles. There is a remarkable difference between rabbit blood and human or dog blood, and there is also a great difference in amount of fat constituents of whole blood and plasma in rabbits and in human beings or dogs. Of the fat constituents of rabbit plasma, cholesterol is one-third of that of human beings, lecithin one-half, and total fatty acids about two-thirds of human beings, while the difference of corpuscle fats is very small. From these facts, the ratios $\frac{\text{total fatty acids}}{\text{lecithin}}$ and $\frac{\text{lecithin}}{\text{cholesterol}}$ in plasma are larger in rabbits than in human beings.

Rabbits as well as human beings and dogs have a racial and individual constancy of fatty substances in blood. All the fat constituents in plasma and corpuscles of rabbits fed carrots and of rabbits fed sunflower seeds on successive days and at few weeks' intervals are shown to be mostly within a very small limit of variation from the individual average and also from the 1st day's value.

The ratios $\frac{\text{total fatty acids}}{\text{lecithin}}$ and $\frac{\text{lecithin}}{\text{cholesterol}}$ are also very close to the racial and individual average and to the 1st day's amount.

It is definitely shown in this experiment that the blood fat values are practically stable, even when 10 cc. of blood are taken every day, if the duration of bleeding lies within 3 to 4 days. This is particularly so on the fat-free diet. When 10 cc. of blood are drawn every day for a longer period, however, changes in amount of fat constituents take place which are reported in the following paper.

STUDIES ON BLOOD FAT.

II. LIPEMIA IN ACUTE ANEMIA.

BY YAJIRO HORIUCHI.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

(Received for publication, July 29, 1920.)

INTRODUCTION.

Experimental lipemia, first described by Boggs and Morris,[1] was produced by them in rabbits by drawing from 15 to 45 cc. of blood every day. To produce the milky plasma 8 to 16 days were required. The blood fats increased from 0.3 to 4.53 per cent, as determined by the Soxhlet extraction method, lecithin being about 10 per cent of the total lipoid. Sakai[2] did not find alimentary lipemia in normal rabbits, even in one from which 20 cc. of blood were drawn and to which 17 gm. of palmin were given but an increase of blood fat in a rabbit which was made anemic by drawing 13 to 20 cc. of blood successively for 5 days, 3 hours after 17 gm. of palmin were given. He examined also the alimentary lipemia in anemic rabbits fed on milk, bread, or ordinary food. In his cases, the fatty acids increased to a high degree, but cholesterol to a less extent, the relation of fatty acids to cholesterol being 12:1 or 16:1. He used the Kumagawa-Suto-Shimidzu method for the determination of fatty acids and cholesterol, and Autenrieth-Funk's colorimetric method for the control of cholesterol estimation. Owing to the need of a large amount of blood in making the analysis of fats by the old methods, the work of these authors was limited, as they could work on the animals only occasionally.

As I considered it probable that a single drawing of a large amount of blood could cause a strong lipemia in a rabbit not previously anemic, I made preliminary experiments by repeated drawing of small amounts of blood from two rabbits, one on sunflower seed diet, the other on carrot diet. In both of them, very high grade lipemia resulted. In further experiments with three rabbits on a fat diet, two rabbits on a fat-free diet, and one on a fat-free diet but with occasional fat food, a very high lipemia was produced and the relation of lipemia to different kinds of foods was carefully studied.

---

[1] Boggs, T. R., and Morris, R. S., *J. Exp. Med.*, 1909, xi, 553.

[2] Sakai, S., *Biochem. Z.*, 1914, lxii, 387.

A few remarks are added as to the methods of blood fat determination. 3 cc. of whole blood and plasma, and 2.8 to 1.2 cc. of corpuscles, obtained from 7 (10–3) cc. of whole blood, were used for extraction. To estimate the lipoid of each blood sample, an appropriate quantity of the extract, according to the degree of lipemia, was used for comparison with the standard solution. For the total fatty acids, 15, 10, 5, 3, 2, sometimes even 1 cc. of whole blood and plasma extract were enough to compare with 5 cc. of the standard solution, while 15 cc. of corpuscle extract were required to compare with 3 cc. of the standard solution. For lecithin, 16, 7, or 5 cc. of whole blood and plasma extract could be compared with the standard direct, while 10 to 15 cc. of corpuscle extract were always used. In the cholesterol determination, 5 to 15 cc. of whole blood and plasma extract were taken to compare with 3 to 5 cc. of the standard, while only 2 to 3 cc. of standard solution were needed for the 15 cc. of corpuscle extract.

Change in the Blood Fats of a Rabbit, when About 10 Cc. of Blood Are Drawn Daily for 15 Days.

A. Lipemia of a Rabbit on a Fat Diet.—About 10 cc. of blood were drawn from the ear every day from a relatively small rabbit (No. 10), weighing 1,190 gm., while being fed with sunflower seeds. For the first 3 days of bleeding the composition of the blood remained unchanged. On the 4th day (72 hours after the first bleeding), namely after 35 cc. of blood were taken, the blood underwent a characteristic change, whole blood becoming like chocolate cream with a creamy plasma. This appearance became more marked on the following day. As shown by the analysis of the blood fats, total fatty acids, lecithin, and cholesterol increased greatly in the whole blood and plasma, particularly in plasma. The highest values for total fatty acids of the whole blood and plasma were 7.09 and 8.41 times the value in the first specimen. Lecithin and cholesterol increased in the whole blood and plasma, but not so much as total fatty acids. The greatest increase in the whole blood and plasma for lecithin was 2.48 and 3.63 times the 1st day's value, cholesterol being 3.00 and 4.00 times the original amounts. Fats in the corpuscles were slightly increased, but the increased amounts were within the limit of analytical error.

B. Lipemia of a Rabbit on a Fat-Free Diet.—The same experiment was performed on a larger rabbit, weighing 1,980 gm., on a fat-free carrot diet. For the first 7 days, the blood fats showed no particular change, although 10 cc. of blood were drawn every day, and the percentage of corpuscles to the whole blood decreased from 37 to 19, and hemoglobin (Sahli) from 55 to 30. For 2 days thereafter, blood fat constituents increased gradually. Finally, a large amount of blood (45 cc.) was drawn, which caused a strong lipemia lasting for 3 days. On the 2nd day, the lipemia was greatest, and the increase of total fatty acids in whole blood and plasma was 9.53 and 15.52 times the normal value, lecithin being 4.06 and 7.50, cholesterol 2.20 and 3.43 times the normal. Fat in the corpuscles was not much changed.

The ratios $\frac{\text{total fatty acids}}{\text{lecithin}}$ and $\frac{\text{lecithin}}{\text{cholesterol}}$ increased greatly on the 1st day of lipemia in Experiment B as well as in Experiment A.

The analytical results of Experiments A and B, and the degree of increase of blood fat compared with the first sample are given in Table I.

In one case (a rabbit fed on sunflower seed) strong lipemia was produced easily by repeated small bleedings, but in the other (a rabbit fed on carrots) an extensive additional bleeding was required. Also the total fatty acids increased to a greater extent than lecithin or cholesterol, and the lipemia in both cases is that of plasma but not that of corpuscles.

As the only difference between Experiments A and B, excepting the size of the animals, was in the feeding of a fatty and of a fat-free food, it is very probable that the fat of lipemia in Experiment A is due to the food fat, and in Experiment B to the mobil-

TABLE I.

Lipemia of Rabbits Produced by Repeated Bleeding.

Rabbit No.	Date.	Weight.	Blood drawn.	Hemoglobin.	Plasma.	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
							Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
A. Lipemia on a fat diet.																					
	1918	*gm.*	*cc.*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						
10	Nov. 11	1,190	10		Clear.	32	0.34	0.34	0.31	0.21	0.16	0.31	0.12	0.10	0.18	1.62	2.13	1.00	1.75	1.60	1.72
	" 12		10		"	22	0.36	0.34	0.37	0.20	0.17	0.35	0.11	0.12	0.22	1.80	2.00	1.06	1.82	1.42	1.59
	" 13		15		"	22	0.39	0.35	0.36	0.21	0.18	0.30	0.14	0.14	0.15	1.86	1.94	1.20	1.50	1.29	2.00
	" 14		10		Very strongly milky.	18	1.95	2.32	0.43	0.32	0.32	0.38	0.30	0.30	0.17	6.99	7.25	1.13	1.07	1.07	2.24
	" 15		10		" " "	19	2.41	2.86	0.51	0.52	0.58	0.32	0.36	0.40	0.19	4.63	4.93	1.59	1.44	1.45	1.68
Ratios of highest fat to the original value estimated as 1.00..							7.09	8.41	1.65	2.48	3.63	1.23	3.00	4.00	1.22	3.76	3.40	1.59	1.04	0.91	1.30
B. Lipemia on a fat-free diet.																					
	1919																				
17	Feb. 15	1,980	10	55	Clear.	37	0.30	0.21	0.31	0.18	0.10	0.36	0.10	0.07	0.14	1.67	2.10	0.86	1.80	1.43	2.57
	" 16		10	50	"	30	0.24	0.21	0.36	0.19	0.10	0.36	0.10	0.06	0.16	1.26	2.10	1.00	1.90	1.67	2.25
	" 17	1,955	10	45	"	26	0.22	0.18	0.35	0.16	0.08	0.35	0.10	0.08	0.13	1.38	2.25	1.00	1.60	1.00	2.69
	" 18		10	42	"	26	0.22	0.17	0.40	0.15	0.08	0.32	0.09	0.08	0.12	1.47	2.13	1.25	1.67	1.00	2.67
	" 19		10	32	"	19	0.19	0.16	0.44	0.16	0.08	0.41	0.09	0.07	0.11	1.19	2.00	1.07	1.78	1.14	3.73
	" 20		10	30	"	19	0.22	0.20	0.38	0.18	0.11	0.52	0.10	0.08	0.13	1.22	1.82	0.73	1.80	1.38	4.00
	" 21	1,910	10	30	"	21	0.27	0.24	0.35	0.22	0.12	0.50	0.12	0.11	0.16	1.23	2.00	0.70	1.83	1.09	3.13
	" 22		10	30	"	23	0.29	0.30	0.29	0.27	0.16	0.56	0.12	0.10	0.21	1.07	1.88	0.52	2.25	1.60	2.67

	Feb. 23	1,910	*45*	32	Clear.	27	0.34	0.32	0.42	0.27	0.15	0.62	0.11	0.10	0.13	1.26	2.13	0.68	2.45	1.50	4.77
	" 24	1,880	10	23	Very strongly milky.	20	2.79	3.07	0.46	0.48	0.47	0.60	0.17	0.18	0.16	5.81	6.53	0.77	2.82	2.61	3.75
	" 25		10	25	" " "	23	2.86	3.26	0.50	0.73	0.75	0.58	0.22	0.24	0.14	3.92	4.35	0.86	3.32	3.13	4.14
	" 26	1,865	10	26	Strongly milky.	26	1.83	2.20	0.38	0.52	0.50	0.57	0.21	0.22	0.14	3.52	4.40	0.67	2.48	2.27	4.07
	" 27		10	29	Slightly cloudy.	24	0.49	0.51	0.36	0.25	0.16	0.55	0.16	0.17	0.12	1.96	3.19	0.65	1.56	0.94	4.58
	" 28	1,900	10	30	Clear.	27	0.49	0.52	0.34	0.22	0.15	0.47	0.15	0.15	0.13	2.23	3.47	0.72	1.47	1.00	3.62
	Mar. 3		10	42	"	34	0.46	0.45	0.47	0.21	0.13	0.40	0.14	0.13	0.15	2.19	3.46	1.18	1.50	1.00	2.67
	" 6	1,925	10	47	"	34	0.33	0.34	0.47	0.27	0.11	0.59	0.12	0.10	0.13	1.22	3.09	0.80	2.25	1.10	4.54
	" 22	1,970	10	55	"	39	0.29	0.20	0.42	0.26	0.10	0.46	0.11	0.07	0.15	1.12	2.00	0.91	2.36	1.43	3.07
Ratio of highest fat to the original value estimated as 1.00							9.53	15.52	1.61	4.06	7.50	1.72	2.20	3.43	1.50	3.48	3.11	1.45	1.84	2.19	1.86

ized fat from the tissues. Further, the above fact proves that the lipemia caused by inability to utilize food fat occurs more easily and earlier than the lipemia from the stored fat. And again, larger hemorrhage is probably necessary to cause the mobilization of the fat in tissues, and also a certain period must elapse before the fat will appear in the blood stream. Moreover, we learn that it is absolutely necessary to avoid excessive hemorrhage from rabbits not merely in the study of fat metabolism but also in ordinary chemical and serological work, as the content of blood would be changed. In the above two cases, the fat constituents in blood remained unchanged for 3 days in Experiment A and 7 days in Experiment B, when 10 cc. of blood were taken every day.

Does Acute Anemia Cause Lipemia in a Rabbit?

In this experiment, a large amount of blood (45 cc.) was drawn from each rabbit. In Experiment A with sunflower seeds, a slightly cloudy plasma was observed after 24 hours. The typical chocolate cream-like whole blood and creamy plasma were seen after 48 hours, and the lipemia reached the maximum on the following day.

In Experiment B with carrots, the fat constituents in plasma and whole blood increased somewhat after 24 hours. The plasma on the next day became milky, but began to clear again on the 4th day.

The analytical results of blood fats in Experiment A show a large increase of total fatty acids in plasma and a proportional increase in whole blood. Lecithin and cholesterol increased also in plasma, and therefore in whole blood, but not so much as total fatty acids. In Experiment B, the greatest increase of total fatty acids in plasma took place 48 hours after drawing the blood. It was about four times the first sample. As in Experiment A, lecithin and cholesterol increased markedly in plasma, but not so much as the total fatty acids. Fat values in corpuscles in both cases are not particularly increased. Therefore, the lipemia of both rabbits, occurring markedly from food fats and less so from tissue fats, was in plasma alone, and not in corpuscles.

TABLE II.

Lipemia of Rabbits Produced by Acute Anemia.

Rabbit. No.	Date.	Weight.	Blood drawn.	Hemoglobin.	Plasma.	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
							Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
A. Lipemia of a rabbit fed sunflower seed.																					
	1919	*gm.*	*cc.*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						
14	Jan. 17	1,675	*45*	60	Clear.	37	0.30	0.32	0.25	0.25	0.14	0.43	0.12	0.06	0.20	1.20	2.29	0.58	2.08	2.33	2.15
	" 18		10	26	Slightly cloudy.	21	0.79	0.79	0.47	0.29	0.19	0.48	0.11	0.10	0.23	3.29	4.16	0.98	2.64	1.90	2.09
	" 19		10	15	Very strongly milky.	12	2.23	2.43	0.55	0.46	0.46	0.75	0.26	0.27	0.20	4.85	5.28	0.73	1.77	1.70	3.75
	" 20		10	13	" " "	13	3.59	4.02	0.55	0.64	0.65	0.66	0.36	0.39	0.17	5.61	6.18	0.83	1.78	1.67	3.88
Ratio of the highest fat to the original value							11.97	12.56	2.20	2.56	4.64	1.74	3.00	6.50	1.15	4.68	2.70	1.69	1.27	0.82	1.80
B. Lipemia of a carrot-fed rabbit																					
16	Feb. 6	2,165	*45*	55	Clear.	37	0.32	0.28	0.34	0.21	0.11	0.38	0.11	0.06	0.18	1.52	2.55	0.89	1.91	1.83	2.11
	" 7		10	22	"	21	0.37	0.38	0.27	0.25	0.20	0.38	0.12	0.09	0.22	1.48	1.90	0.71	2.08	2.22	1.73
	" 8		10	22	Milky.	17	1.09	1.21	0.32	0.30	0.28	0.46	0.15	0.14	0.17	3.63	4.32	0.70	2.00	2.00	2.71
	" 9		10	22	Slightly milky.	15	0.68	0.71	0.36	0.29	0.25	0.59	0.13	0.12	0.20	2.34	2.84	0.61	2.23	2.08	2.95
	" 12		10	30	Clear.	28	0.38	0.36	0.38	0.23	0.15	0.42	0.12	0.09	0.18	1.65	2.40	0.90	1.92	1.67	2.33
Ratio of the highest fat to the original value							3.41	4.32	1.12	1.43	2.55	1.55	1.36	2.33	1.22	2.39	1.69	1.01	1.17	1.21	1.40

To make clear the degree of increase of blood fats in plasma by acute anemia, a summary of Table II is added.

Summary of Table II.

Day of anemia.	Ratio of the high fat value in plasma to the original amount in lipemia by acute anemia.		
	Total fatty acids.	Lecithin.	Cholesterol.
	A. No. 14 (sunflower seed diet).		
1st	1.00	1.00	1.00
2nd	2.47	1.36	1.67
3rd	7.59	3.29	4.50
4th	12.56	4.64	6.50
	B. No. 16 (carrot diet).		
1st	1.00	1.00	1.00
2nd	1.36	1.82	1.50
3rd	4.32	2.55	2.33
4th	2.54	2.27	2.00
7th	1.29	1.36	1.50

The fat value of the 1st day's blood is called 1.00; the other numbers express the relation of high fat values to the originals.

How Long Does the Lipemia Produced by Acute Anemia Continue?

In this experiment, three rabbits, two fed on sunflower seeds and one on carrots, were used. It was desired to study the whole course of the lipemia of acute anemia.

Experiment A.—One of the two rabbits on fatty food was made anemic by drawing a large amount of blood (70 cc.), in which the milky plasma, beginning slightly after 24 hours as in previous cases, developed strong milkiness, reached its maximum after 72 hours, and then began to decrease on the next day. The milkiness disappeared from the plasma 168 hours after the first bleeding and the increased fat constituents, total fatty acids, lecithin, and cholesterol, in plasma had returned to about the original amount after 18 days.

In Rabbit 18, which had been used 2 months before as No. 16 for a similar experiment, the lipemia was of a little different type as regards its beginning and duration. The marked lipemia began 24 hours after the first drawing of blood and reached the maximum 48 hours after the first bleeding. Strongly milky plasma was seen for 5 days. Moreover, the lipemia was more marked than in others, the fat value being the highest of all the cases; highest total fatty acids being as large as 25.48 times the 1st day's amount. The other lipoid constituents in plasma were also very high. The lipemia gradually diminished and after 17 days all the fat values were near the original amount.

Experiment B.—With Rabbit 19 (carrot-fed), as the drawing of a relatively small amount of blood (35 cc.) caused very little difference in the blood fat constituents, another bleeding of 25 cc. was made on the next day. Then the increase of fats in plasma began promptly, and milkiness continued for the 2 following days. However, in this carrot-fed rabbit as well as in the previous one on the same diet, the change in fat constituents was relatively small. Although the total fatty acids, lecithin, and cholesterol decreased day by day, they were a little higher than the first amounts even after 14 days.

The summary, calculated from Table III, shows the percentage increase of fats in plasma, during the whole course of the lipemia.

In the three cases, the milky plasma continued for 6, 5, and 3 days, respectively, after the first hemorrhage, while each blood fat constituent was generally higher than the original amount even after 17, 14, and 13 days, respectively.

TABLE III.

Whole Course of Lipemia of Rabbits Produced by Acute Anemia.

Rabbit No.	Date.	Weight.	Blood drawn.	Hemoglobin.	Plasma.	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Total fatty acids / Lecithin			Lecithin / Cholesterol		
							Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
A. Lipemia on fat diet.																					
	1918	*gm.*	*cc.*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						
13	Dec. 31	2,330	*70*		Clear.	38	0.32	0.26	0.50	0.25	0.09	0.47	0.10	0.06	0.14	1.28	2.89	1.06	2.50	1.50	3.36
	1919																				
	Jan. 1	2,270	10		Slightly cloudy.	26	0.55	0.55	0.51	0.21	0.11	0.51	0.11	0.08	0.17	2.62	5.00	1.00	1.91	1.38	3.00
	" 2		10		Very strongly milky.	24	3.12	3.93	0.56	0.56	0.55	0.51	0.22	0.23	0.22	5.57	7.15	1.10	2.55	2.39	2.32
	" 3	2,200	10		" " "	20	4.16	5.19	0.56	0.68	0.70	0.59	0.34	0.37	0.19	6.12	7.41	0.95	1.71	1.62	3.11
	" 4		10		" " "	27	2.28	3.05	0.50	0.48	0.42	0.54	0.21	0.20	0.20	4.75	7.26	0.93	2.29	2.10	2.70
	" 5		10		Milky.	33	0.99	1.30	0.53	0.35	0.24	0.56	0.15	0.12	0.18	2.83	5.42	0.95	2.33	2.00	3.11
	" 6	2,230	10		Slightly cloudy.	30	0.70	0.73	0.48	0.28	0.19	0.52	0.13	0.11	0.18	2.50	3.84	0.92	2.15	1.73	2.89
	" 7		10		Clear.	31	0.42	0.40	0.53	0.26	0.18	0.53	0.13	0.11	0.18	1.62	2.22	1.00	2.00	1.64	2.94
	" 17	2,280	10		"	32	0.29	0.28	0.40	0.23	0.13	0.52	0.13	0.10	0.19	1.26	2.15	0.77	1.77	1.30	2.74
Ratio of the highest fat to the original value							13.00	19.96	1.12	2.72	7.78	1.26	3.40	6.17	1.57	4.78	2.56	1.04	1.04	1.59	0.93
18	Mar. 15	2,380	*45*	60	Clear.	38	0.24	0.21	0.29	0.29	0.12	0.56	0.12	0.06	0.18	0.83	1.75	0.50	2.42	2.00	3.11
	" 16		10	26	Milky.	23	1.92	2.17	0.44	0.35	0.30	0.55	0.22	0.23	0.17	5.49	7.23	0.80	1.59	1.30	3.24
	" 17	2,335	10	30	Very strongly milky.	21	4.43	5.35	0.35	0.70	0.74	0.57	0.44	0.50	0.17	6.33	7.23	0.61	1.59	1.48	3.35
	" 18		10	32	" " "	23	2.68	3.30	0.51	0.53	0.48	0.61	0.38	0.44	0.15	5.06	6.88	0.84	1.39	1.09	4.07
	" 19	2,310	10	30	Strongly milky.	25	2.39	2.95	0.43	0.49	0.45	0.60	0.28	0.30	0.14	4.88	6.56	0.72	1.75	1.50	4.29
	" 20		10	32	Milky.	24	1.82	2.25	0.32	0.35	0.32	0.58	0.23	0.25	0.18	5.20	7.03	0.55	1.52	1.28	3.22
	" 23	2,325	10	37	Clear.	28	0.38	0.38	0.30	0.29	0.17	0.82	0.14	0.12	0.18	1.31	2.24	0.37	2.07	1.42	4.56
	" 26	2,330	10	47	"	26	0.31	0.29	0.39	0.28	0.16	0.84	0.13	0.11	0.21	1.11	1.81	0.46	2.16	1.45	4.00
	Apr. 1	2,350	10	43	"	36	0.29	0.21	0.37	0.27	0.10	0.62	0.12	0.09	0.22	1.07	2.10	0.60	2.25	1.11	2.82
Ratio of the highest fat to the original value							18.46	25.48	1.76	2.41	6.17	1.50	3.67	8.33	1.22	7.63	4.13	1.68	0.93	0.75	1.47

B. Lipemia on fat-free diet.

19	Apr. 10	1,750	*35*	55	Clear.	41	0.37	0.16	0.22	0.22	0.12	0.39	0.12	0.08	0.20	1.68	1.33	0.56	1.83	1.50	1.95
	" 11		*25*	33	"	23	0.21	0.20	0.23	0.20	0.15	0.40	0.11	0.09	0.19	1.05	1.33	0.58	1.82	1.67	2.11
	" 12		10	23	"	23	0.28	0.46	0.30	0.22	0.17	0.39	0.11	0.09	0.17	1.27	2.71	0.77	2.00	1.89	2.29
	" 13		10	22	Slightly milky.	15	1.77	0.93	0.26	0.24	0.21	0.43	0.14	0.13	0.21	3.21	4.43	0.60	1.71	1.62	2.05
	" 14		10	23	Milky.	15	1.19	1.34	0.29	0.32	0.30	0.45	0.16	0.16	0.18	3.72	4.47	0.64	2.00	1.88	2.50
	" 15		10		Slightly cloudy.	18	0.62	0.67	0.34	0.25	0.18	0.60	0.13	0.12	0.19	2.48	3.72	0.57	1.92	1.50	3.16
	" 16		10	25	Clear.	21	0.36	0.38	0.38	0.24	0.15	0.58	0.12	0.10	0.18	1.50	2.53	0.66	2.00	1.50	3.22
	" 19		10	32	"	24	0.40	0.39	0.44	0.24	0.14	0.50	0.11	0.09	0.17	1.67	2.79	0.88	2.18	1.56	2.24
	" 23		10	39	"	30	0.27	0.24	0.36	0.24	0.13	0.45	0.11	0.08	0.20	2.45	1.85	0.65	2.18	1.63	2.25
Ratio of the highest fat to the original value..........							3.22	8.38	2.00	1.45	2.50	1.54	1.33	2.00	1.05	2.21	3.36	1.57	1.19	1.26	1.65

Summary of Table III.

Rabbit No.	Day of anemia.	Ratio of the high fat value in plasma to the original amount through the whole lipemia course.		
		Total fatty acids.	Lecithin.	Cholesterol.
A. Sunflower seed diet.				
13	1st	1.00	1.00	1.00
	2nd	2.12	1.22	1.33
	3rd	15.12	6.11	3.83
	4th	19.96	7.78	6.17
	5th	11.73	4.67	3.33
	6th	5.00	2.67	2.00
	7th	2.81	2.11	1.83
	8th	1.54	2.00	1.83
	18th	1.08	1.44	1.67
18	1st	1.00	1.00	1.00
	2nd	10.33	2.50	3.83
	3rd	25.48	6.17	8.33
	4th	15.71	4.00	7.33
	5th	14.05	3.75	5.00
	6th	10.71	2.67	4.17
	9th	1.81	1.42	2.00
	12th	1.38	1.33	1.83
	15th	1.00	0.83	1.50
B. Carrot diet.				
19	1st	1.00	1.00	1.00
	2nd	1.18	1.25	1.13
	3rd	2.27	1.42	1.13
	4th	5.47	1.75	1.63
	5th	7.88	2.50	2.00
	6th	3.94	1.50	1.50
	7th	2.24	1.25	1.25
	9th	2.29	1.17	1.13
	14th	1.41	1.08	1.00

What Influence Does Fat Feeding Have on the Lipemia of a Rabbit on Carrot Diet in Acute Anemia?

An additional experiment was performed with Rabbit 15, fed on carrots, made lipemic by twice drawing a large amount of blood. 4 days after the first bleeding, and when the blood fat constituents began to decrease, the animal was fed with fatty

TABLE IV.

Influence of Food Fat on the Lipemia of a Rabbit on Fat-Free Diet.

Rabbit No.	Date.	Weight.	Blood drawn.	Hemoglobin	Plasma.	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			$\frac{\text{Total fatty acids}}{\text{Lecithin}}$			$\frac{\text{Lecithin}}{\text{Cholesterol}}$		
							Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
	1919	*gm.*	*cc.*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						
15	Jan. 28	1,600	*40*	60	Clear.	31	0.27	0.25	0.37	0.26	0.13	0.51	0.12	0.10	0.15	1.04	1.92	0.73	2.17	1.30	3.40
	" 29		10	24	Slightly cloudy.	16	0.60	0.66	0.43	0.30	0.27	0.48	0.16	0.14	0.20	2.00	2.44	0.90	1.88	1.93	2.40
	" 30	1,550	*25*	13	Milky.	14	1.01	1.03	0.35	0.35	0.34	0.53	0.20	0.21	0.18	2.89	3.03	0.66	1.75	1.62	2.94
	" 31		10	13	"	12	1.13	1.24	0.49	0.40	0.40	0.44	0.29	0.31	0.17	2.83	3.10	1.11	1.38	1.29	2.59
	Feb. 1	1,540	10	14	"	14	1.05	1.15	0.56	0.37	0.36	0.38	0.22	0.23	0.16	2.84	3.19	1.47	1.68	1.57	2.38
	(Fed sunflower seeds during p.m.)																				
	Feb. 2		10	18	Strongly milky.	17	1.91	2.15	0.34	0.43	0.45	0.42	0.29	0.32	0.14	4.44	4.78	0.81	1.48	1.41	3.00
	" 3	1,550	10	20	Slightly "	19	0.62	0.69	0.56	0.26	0.22	0.38	0.14	0.14	0.14	2.38	3.14	1.47	1.86	1.57	3.71
	" 4		10	22	" "	18	0.59	0.65	0.45	0.26	0.19	0.52	0.14	0.13	0.15	2.27	3.42	0.87	1.86	1.46	3.47
	" 7	1,575	10	26	Clear.	24	0.39	0.28	0.40	0.26	0.20	0.48	0.13	0.12	0.15	1.50	1.40	0.83	2.00	1.67	3.20
Ratio of the highest fat to the original value, before fat diet was given							4.19	4.96	1.51	1.54	3.08	1.04	2.42	3.10	1.33	2.78	1.66	2.01	0.86	1.48	0.86
Ratio of the fat value on the day of fat feeding to the original amount							3.89	4.60	1.51	1.42	2.77	0.75	1.83	2.30	1.07	2.73	1.66	2.01	0.77	1.21	0.70
Ratio of the fat value on the following day of fat feeding to the original value							7.07	8.69	1.92	1.65	3.48	0.82	2.42	3.20	0.93	4.27	2.49	1.11	0.68	1.08	0.88

food (sunflower seeds) half a day. The blood sample of the following morning, instead of diminishing, showed an apparently higher lipemia. The total fatty acids in plasma and whole blood increased nearly twice. Lecithin and cholesterol values also increased. The milkiness of the plasma disappeared soon, and the blood fat constituents decreased also, yet they were a little higher than the original values after 10 days. This experiment clearly indicates the relation between the lipemia from stored fat and the lipemia from food fat in acute anemia. The analytical results are given in Table IV.

SUMMARY AND CONCLUSION.

1. Lipemia, higher than any reported in alimentary lipemia in animals or in the blood of severe human diabetic acidosis, was produced by a single hemorrhage on a rabbit.

In a relatively small rabbit (1,190 gm.), fed with sunflower seeds (fat content about 40 per cent), the repeated drawing of a small amount of blood, amounting to 35 cc. in 3 days, was enough to produce strong lipemia; the highest content of total fatty acids, lecithin, and cholesterol in plasma being 8.41, 3.63, and 4.00 times the original values, respectively, while in the rabbit on fat-free food (carrots which contain 0.4 per cent of fats) the milky plasma appeared only after the repeated drawing of 10 cc. of blood for 9 days and one additional large hemorrhage (45 cc.), the highest amount of the above mentioned fat constituents being on this occasion 15.52, 7.50, and 3.43 times the first plasma fat values.

In the cases of acute anemia, produced by one large hemorrhage (45 cc.) on the fat-fed rabbit, the whole blood looked like chocolate cream and the plasma was thick and creamy. In these cases, the highest amounts of total fatty acids, lecithin, and cholesterol in plasma were found to be 12.56, 4.64, 6.50; 19.96, 7.78, 6.17; and 25.48, 6.17, 8.83 times the normal values, while in carrot-fed rabbits the amounts were 4.32, 2.55, 2.33, and 7.88, 2.50, 2.00 times the normal values.

These lipemias occurred by reason of the inability of the rabbits to utilize the absorbed food fats and mobilized tissue fats in the blood stream. The experiment with Rabbit 15 on carrot diet gives us definite proof that fat feeding increases lipemia to a considerable degree.

2. The duration of the lipemia of acute anemia is relatively short. The lipemia of rabbits on fat begins with slightly cloudy plasma 24 hours after a large bleeding. The creamy or milky plasma appears after another 24 hours and the thickest creamy plasma is observed about 72 hours after the first hemorrhage. Thereafter, the lipemia decreases gradually and disappears within another 72 hours. The cases of carrot-fed rabbits proceeded nearly like rabbits fed on sunflower seeds, the difference being in the amount of fatty contents.

Although the milkiness of plasma disappeared in a relatively short time, the abnormally high values of fats in the blood continued for a markedly long time. High values persisted even after 18 and 14 days from the beginning (Nos. 13, 18, and 19). As the result of small daily blood letting for 9 days and a final large bleeding, Rabbit 17, carrot-fed, had strong lipemia and milky plasma for 5 days. Its blood fat constituents as well as the percentage of corpuscles and hemoglobin were normal in a sample taken 27 days after the plasma began to be milky.

3. The above mentioned lipemia, due either to external food fat or to internal body tissue fat or to both (No. 15), is a case of lipoidemia; *i.e.*, the great increase of total fatty acids was accompanied by a corresponding increase of lecithin and cholesterol. The increase of the latter mentioned substances was, however, not mathematically proportional to that of total fatty acids, and the amounts varied rather irregularly. This lipoidemia of rabbits in acute anemia is a lipoidemia of plasma, but not of corpuscles, as the latter does not share particularly in the fat accumulation in the blood, as can be seen from the analytical results. A similar phenomenon was experienced by Bloor only in the high grade lipemia of some cases of human diabetes mellitus;[3] while in other cases of the same disease and his work[4] on fat assimilation, some increase of the fat constituents in corpuscles was always observed.

4. Lipemia, caused by blood letting, can be produced more easily and in a much higher degree when the fats originate from

---

[3] Bloor, W. R., *J. Biol. Chem.*, 1916, xxvi, 417.

[4] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227, 447.

the food than when they are mobilized from body tissues. It is a fact of some interest that in a carrot-fed rabbit (No. 17) the very striking lipemia was observed after a large blood drawing when the animal had been previously subjected to repeated small bleedings for 9 days amounting to 90 cc. It is possible that the organism had been prepared for mobilization of body fat when previously exposed to anemia for several days.

5. As the increase of total fatty acids is markedly more than that of lecithin and cholesterol, the ratio $\frac{\text{total fatty acids}}{\text{lecithin}}$ in whole blood and plasma is abnormally high in strong lipemia. As a rule the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$ is not particularly changed.

6. As lipemia may be caused both by repeated drawing of small amounts of blood and also by a large hemorrhage, care must be taken in the interpretation of the conclusions from work on blood fats done under such conditions.

7. As to the etiology of lipemia, the decrease of the amount of lipase in blood serum, determined by Sakai by the stalagmometric method of Rona and Michaelis,[5] may be a factor. However, Sakai did not attempt to determine why the lipase in blood serum was decreased. It is very probable that hemorrhage may interfere with the function of the internal organ which is thought to be the seat of the production of lipase—the pancreas—and with the organs in which the lipolytic process[6] takes place, such as the spleen, lymphatic glands, etc., and also the liver[7,8] which is believed to be an important organ for the end or later stages of fat metabolism (Knoop,[9] Leathes, etc.). Accordingly, abnormally lower lipolysis and oxidation of fats may result, and a lipemia may finally be produced. Those important organs for fat metabolism would be gradually weakened for 2 or 3 days after hemorrhage and then gradually recover, as seen in t[illegible]

---

[5] Rona, P., and Michaelis, L., *Biochem. Z.*, 1911, xxxi, 345. Rona [illegible] *Biochem. Z.*, 1911, xxxiii, 413.

[6] Freudenberg, E., *Biochem. Z.*, 1912, xlv, 467.

[7] Leathes, F. B., The fats, London, 2nd edition, 1913.

[8] Shibata, N., *Biochem. Z.*, 1911, xxxvii, 345.

[9] Knoop, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 150.

disappearance of milkiness of plasma and the decrease of blood fat constituents shown by our experiments.

In fat wandering, which has been much discussed in cases of phosphorus and other poisoning, fat transportation from the tissues into the liver occurs when there is a great diminution of glycogen in the liver. In acute anemia, it might be supposed that the liver glycogen is exhausted before a new supply from food (carrots) reaches the organ. However, the appearance of lipemia in acute anemia is due more likely to a temporary fasting, as a large hemorrhage may produce a kind of inanition. Such severe lowering of nutrition may be enough to produce mobilization of stored fat, giving rise to the accumulation of fat in the blood for some length of time by the synchronic lack of lipolytic action. The lipemia may disappear either when the nutrition of organs and tissues again becomes sufficient from food or when the lipolytic power of the blood is completely regenerated. The idea that some of the tissue fats may be easily and others may not be so easily mobilized is plausible. In Rabbit 18, which was exposed previously to anemia, the lipemia by acute anemia occurred more easily and earlier than in a normal one.

8. Leathes'[7] hypothesis that lecithin is a stage through which the fat must pass before it can be utilized in metabolism, was supported by Bloor[4] by the fact that the lecithin value in corpuscles always increased abnormally, when the plasma contained an extra amount of fat, due to the transformation of fatty acids into lecithin by corpuscles. In my experiments, however, it was not possible to observe this, as the lecithin value in corpuscles was not high. If the above mentioned theory is true, there should result a hypothesis that the extent of that function of corpuscles would be limited in acute anemia, and therefore the fat constituents in corpuscles remain almost unchanged, while extra fat is left in the plasma without being taken up by corpuscles, resulting in a condition of lipemia.

A DEFICIENCY IN HEAT-TREATED MILKS.

By AMY L. DANIELS and ROSEMARY LOUGHLIN.

(*From the Department of Nutrition, Child Welfare Research Station, University of Iowa, Iowa City.*)

(Received for publication, August 9, 1920.)

From time to time during the past few years there have appeared in the literature reports of investigations dealing with the biological value of heat-treated milk. These are more or less contradictory, some authors contending that while certain chemical changes have resulted from the processes employed, they have not altered the biological value of the milk.[1] Indeed, in certain instances it has been pointed out that boiled milk of another species is better borne than raw milk. On the other hand, there are experiments which indicate that the nutritive value of raw milk is much greater than that of boiled milk. These varying results may be due (1) to the different methods employed in heating the milk; or (2) to the different lengths of time the experiments were run. Milk boiled quickly may have quite different values from that which has been brought slowly to the boiling temperature; and an experiment continued over many months may produce results which are not discernible in a short period investigation. Rickets is not a quickly developing syndrome, but the outcome of a greater or less deficiency over a considerable period.

Among those who contend that milk has been made less valuable by heating to high temperatures, there is little unanimity of opinion regarding the cause of the deterioration. Recent findings relative to the thermostability of the antiscorbutic vitamine may account for some of the apparent inconsistencies;[2] but, in those cases where the investigations were carried out with rats, it would seem that the biologic impotency cannot be attributed altogether to the destruction of this vitamine, for rats in our laboratory which have been fed purified rations, containing no demonstrable amount of the antiscorbutic vitamine, throughout a complete life cycle have made what is considered normal growth; have reproduced; and reared their young. These, in turn, have repeated the performance of the parents.

---

[1] The literature has been reviewed by Lane-Claypon, J. E., Milk and its hygienic relations, London, New York, Bombay, and Calcutta, 1916, 225.

[2] Chick, H., Hume, M. E., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131. Hess, A. F., and Fish, M., *Am. J. Dis. Child.*, 1914, viii, 385. Hess, A. F., *Am. J. Dis. Child.*, 1916, xii, 152.

Other substances which have been considered as possibly responsible for the lack of physiologic well being of animals fed heat-treated milk are the casein, and the antineuritic vitamine (water-soluble B). McCollum and Davis[3] fed rations in which the sole source of the water-soluble vitamine was superheated whey (15 pounds pressure for 1 hour) in certain cases; and in others the water-alcohol extract of wheat embryo similarly treated. There was no evidence that the vitamine was destroyed. The rats grew quite as well as control animals receiving similar rations containing the unheated materials. When, however, skim milk powder, heated for a considerable period in a double boiler, or for 1 hour in an autoclave at 15 pounds pressure, was used, growth was not comparable to that on the unheated powder. This heated milk powder also lost its potency as a supplementing material for rations consisting of polished rice, salts, and butter fat—rations which require both protein and the water-soluble food accessory to make them support growth. The addition of a growth minimum (10 per cent) of unheated casein to a ration consisting of superheated milk powder stimulated growth, which continued slowly throughout the experiment. The authors conclude that heating casein for 1 hour in an autoclave at 15 pounds pressure quite destroys its biologic value as a complete protein.

Hogan,[4] on the other hand, believes that high temperatures affect the vitamines rather than the proteins. He found that rations including superheated casein and egg white, as essential parts of the protein requirement, produced growth similar to that secured by rations containing the unheated proteins. In a second group of experiments, when corn mixtures, which furnished the vitamines, were superheated before the addition of these proteins, growth was less marked than on similar rations which were unheated. Protein deterioration could not have been responsible for the lack of growth here. The vitamines appear to be at fault. There is, however, no experimental evidence to indicate which of the vitamines may have been affected.

---

[3] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

[4] Hogan, A. G., *J. Biol. Chem.*, 1917, xxx, 115.

Gibson and Concepción[5] fed fowls a diet of polished rice and milk, some being given raw milk (100 cc.), while others received an equal amount of rice and autoclaved milk. The results indicate that milk has little protective action against polyneuritis, for the birds receiving these additional milks developed polyneuritis in about the same time as those fed polished rice alone. The autoclaved milk, however, did not appear to promote the onset of the neuritic symptoms. When a considerably larger amount (200 cc.) of milk, either fresh or autoclaved, was fed, neither group developed neuritis and there was no evidence of degenerative changes in the peripheral nerves of these birds. From these results it would seem that the antineuritic vitamine had not been materially affected by the high temperatures (2 hours at 125°C.). Comparable results were also obtained with pigs and dogs.

Recently, some suspicion has been cast on the thermostability of the fat-soluble vitamine in certain fats. Osborne and Mendel[6] allowed steam to pass through butter oil for 2½ hours. This, when used as the source of fat-soluble A in rations, gave every indication of being as efficient as the untreated butter oil. Steenbock and coworkers,[7] however, have reported that the fat-soluble vitamine is readily destroyed even below 100°C. It is probable that the apparent inconsistency in the results of these investigations lies in the fact that in one case the fat was heated in the presence of water (steam), whereas in the other no moisture was included. We have found no reports dealing with the effects of superheat on the fat-soluble complex as it exists in milk.

---

[5] Gibson, R. B., and Concepción, I., *Philippine J. Sc.*, *Section B*, 1916, xi, 119.

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxiv, 37.

[7] Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.

In a recent study (1916) of the nutritive value of milk held at the boiling temperature for different periods, Daniels and Stuessy[8] observed that rats fed milk boiled 1, 10, and 45 minutes, respectively, grew slowly, failed to achieve the expected weight for normal animals, and never reproduced. Growth curves of rats receiving milk heated above 100°C. (114°C. for 45 minutes) were fairly comparable to those receiving milk held at the boiling temperature, nutrition disaster intervening only somewhat earlier. The addition of well washed coagulated egg yolk and casein to the diets of those animals receiving the milk held at the boiling temperature for 45 minutes, and of coagulated egg white to the superheated milk, caused growth to be resumed. The animals fed the egg yolk additions produced several litters of young which were successfully suckled. Although the authors offer in explanation of their results the possible destruction of some of the casein, which was supplemented by the added protein, they suggest that there might be other contributing factors, such as the vitamines and inorganic constituents which were responsible for the growth stimulation.

Since the first report, some of the above experiments have been repeated with the view of determining, if possible, what substance or substances are changed by the heating processes. In the earlier work, in order that all conditions during heating might be as nearly comparable as possible, equal quantities of milk (1 pint) were brought to the desired temperature in glass containers of the same size and form, which were completely surrounded by cold water. The period of boiling was counted from the time the milk reached the boiling temperature (98°F.), the time required to bring the milk to the boiling point (about 35 minutes) not being considered. In the new experiments, since the length of time the milk was held at the boiling temperature appeared to make but little difference in the growth of the animals, the milk was brought quickly to the boiling point in an open aluminum kettle, and the boiling continued for just 1 minute. At that time it was believed that milk brought quickly to the boiling point was in every way comparable to that heated by the former method. In these experiments special care was taken to select well nourished young rats from our stock group. Our rats of the first generation fed this quickly heated milk grew normally, and to all appearances were perfectly nourished animals (Chart 1). Growth in these rats was in such striking contrast to that of the earlier work that it seemed best to repeat the former experiments, heating the milk under the same conditions as before. Our results with this second group of rats fed the milk brought slowly to the boiling point were the same as those of the first experiments—the animals grew slowly, reached only about half the normal size, and failed to reproduce (Chart 2). A comparison of the growth curves of these milk-fed animals can lead to but one conclusion; namely, that milk brought quickly to the boiling temperature and held there for only a short period is little affected, while that which has been heated for a considerable period (35 minutes), even below the boiling temperature, is so changed that it fails to meet the nutritive requirements of rats.

---

[8] Daniels, A. L., and Stuessy, S., *Am. J. Dis. Child.*, 1916, xi, 45.

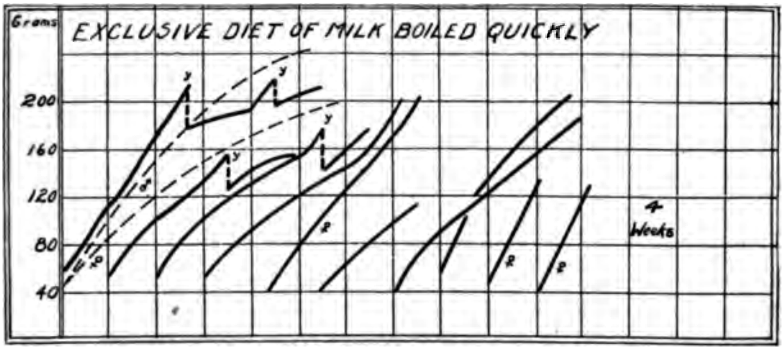


Chart 1. These animals were fed milk heated *quickly* to the boiling temperature and sustained for 1 minute.

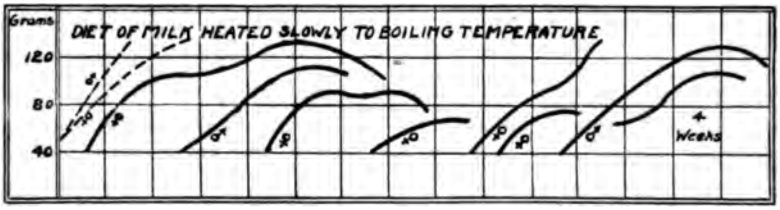


Chart 2. Rats fed milk heated slowly to the boiling temperature grew at about half the usual rate.

The results with the slowly heated milk led us to investigate the effects of commercially canned milk and milk pasteurized by the "hold" system—a system used extensively in dairies and in institutions where numbers of babies are cared for. Previous experience in our laboratory with milk pasteurized by the "flash" system has indicated that it is little affected by the process. Rats grew as well on this as on milk raised quickly to the boiling temperature.

The pasteurized milk used in our experiments was prepared under the same conditions as that used for the babies in our children's hospital. The milk was placed in individual nursing bottles which were surrounded by cold water. The water was heated by steam to 65°C. in some cases, and in others to 82°C., the time required for heating varying from 30 to 45 minutes, depending upon the pressure on the particular day. The milk was then held at the desired temperature 40 minutes, after which it was cooled by running water and placed on ice.[9]

In our experiments with commercially canned milk, both the sweetened (condensed) and the unsweetened (evaporated) were used. In the latter case (evaporated milk) three brands, designated as Brands A, B, and C, were tested. In one case only were we able to get definite information regarding the methods of preparation. We infer that the methods employed in the other two cases were similar. The process consists in holding the milk at the boiling temperature for approximately 10 minutes. It is then evaporated *in vacuo* at a temperature of 130–140° F. until the ratio is approximately two to one. After cooling, it is canned, sealed, and sterilized at 240° F. for about 20 minutes. These milks diluted with equal quantities of distilled water, to which a few specks of iron citrate were added, were fed *ad libitum*, especial care being taken to see that an abundance of milk was always provided.

In the production of the condensed milk tested according to the statement of the manufacturer, after adding the sugar the milk is first heated to approximately 200° F. This is then condensed to the desired consistency in vacuum pans, the temperature during this part of the operation averaging 150° F., but as the milk condenses the temperature gradually drops until it reaches about 120° at the completion of the process. This milk was fed undiluted, distilled water being provided in other containers.

---

[9] We appreciate that the temperature of pasteurization was somewhat higher than that used in the commercial process, and therefore no conclusions regarding the biologic value of the commercially pasteurized milk can be drawn. Experiments with milk commercially pasteurized are now in progress.

The growth curves of the rats fed the pasteurized milk (Chart 3) are very similar to those of animals which received the milk heated slowly to the boiling temperature. As in the latter case, the rats grew at about half the usual rate and never attained the normal size for adult animals.

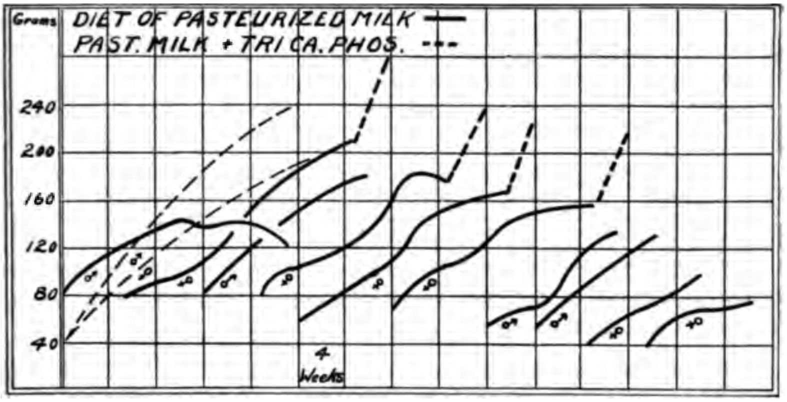


Chart 3. Growth curves of rats fed milk pasteurized by the "hold" process in the apparatus used for pasteurizing the hospital infants' milk feedings. During Period 2 (represented by the broken line) the addition of tricalcium phosphate incorporated in a starch paste stimulated growth.

With the unsweetened (evaporated) milk the results were even more surprising (Chart 4). On Brand A the animals made almost no growth gains and died after a few weeks. On Brands B and C slightly better results were obtained. The animals gained slowly and lived for somewhat longer periods, but all ultimately died in a miserable condition—emaciated, with roughened coats, but with no signs of xerophthalmia. The somewhat better results with Brands B and C may possibly be explained by the fact that the animals on these were about 2 weeks older when the experiment was begun.

The animals fed the sweetened (condensed) milk[10] made decidedly better growth than any of our rats fed the other forms of long heat-treated milk; in fact their curves of growth are fairly comparable to those of normal animals (Chart 5, Group 2). Although the temperatures used in the process of condensing,

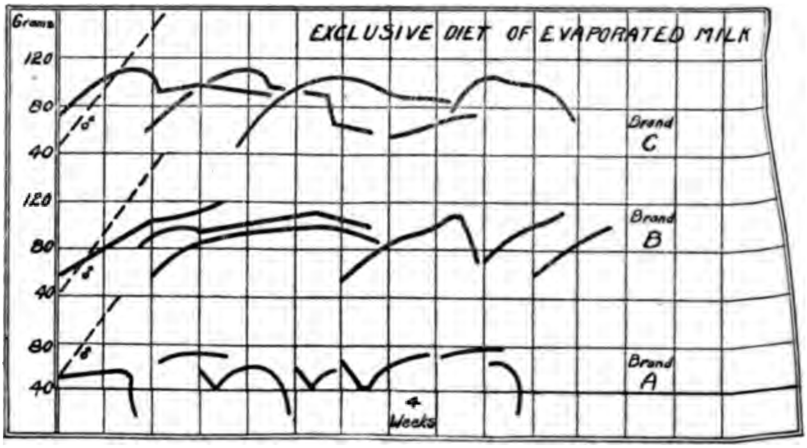


CHART 4. Animals fed evaporated milk (unsweetened) made scarcely no growth and died after a few weeks.

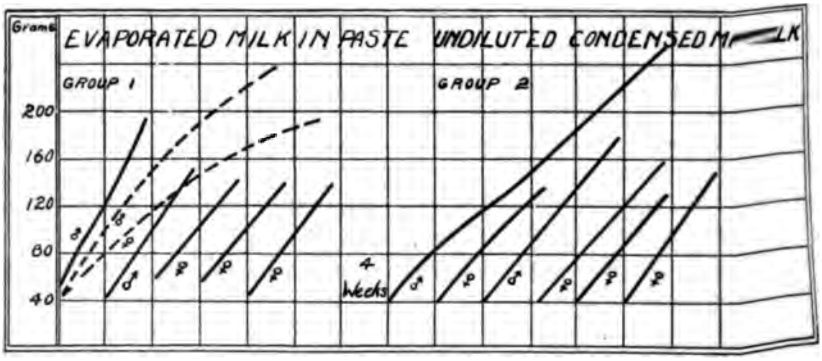


CHART 5. Group 1 indicates the rate of growth of animals fed evaporated milk made into a starch paste.

Group 2 are growth curves of animals fed undiluted condensed (sweetened) milk.

---

[10] We are indebted to Miss Emma Francis, Battle Creek, Michigan, for assistance in these experiments.

after the initial step, were not so very different from those employed in our pasteurization processes, the chemical changes produced, seemingly, had not taken place. In view of our other findings these results were at first confusing. The explanation, however, was forthcoming later in the investigation.

In studying what changes had taken place in the unsweetened (evaporated) and our long heat-treated milks, our attention was first directed toward the possible destruction of the two vitamines, fat-soluble A and water-soluble B. The addition of liberal amounts (25 cc. per 200 cc. of milk) of the water-alcohol extract of wheat embryo to the milk raised slowly to the boiling temperature produced no perceptible change in the growth curves of these animals. Nor was there any evidence that the antineuritic vitamine was affected in the milk heated to the higher temperatures (evaporated milk). Those animals fed purified rations[11] in which the antineuritic material was supplied by fresh milk in one case and diluted evaporated milk in another, gave no indication that the antineuritic vitamine was destroyed by the condensing process (Chart 6). During the early part of the investigation too little milk (less than 200 cc. per 100 gm. of ration) was added to produce normal growth. The growth curves of the animals receiving the evaporated milk additions, however, were consistently better than those of animals receiving the fresh milk mixture. These better results seem to point to a higher antineuritic content of the food mixture containing the evaporated milk. In preparing the foods especial care was taken to dilute the evaporated milk with equal quantities of distilled water. A comparison of the protein (N × 6.25) content of this diluted milk (3.5 per cent) and the fresh (3.3 per cent) milk suggested that the evaporated milk, as fed, might contain slightly more of the vitamine. We appreciate, however, that the amount of protein

---

[11] The purified rations consisted of:

Casein	13.5 gm.
Corn-starch	77.0 "
Suitable salt mixtures	6.0 "
Milk, evaporated	28.0 cc.
" fresh	56.0 "

The precipitated casein was washed 24 hours in running water, dissolved in 0.2 per cent sodium hydroxide solution, reprecipitated with diluted acetic acid, and again washed 24 hours in running water.

a milk contains can be, at best, only a very rough estimate of its antineuritic value. Nevertheless, if the antineuritic vitamine had been considerably destroyed by the prolonged heat treatment employed in the evaporation processes, it would have been evidenced, we believe, by a slower growth rate of the animals fed the evaporated milk ration.

We have been unable also to obtain data indicating that the fat-soluble vitamine is appreciably affected, if at all, by the heat treatment of milk. The addition of butter oil (2 gm. per 100 cc. of diluted milk) was without effect in stimulating growth in the stunted animals fed the superheated (evaporated) milk. A comparison of the growth curves of animals fed a purified ration in which the fat-soluble vitamine was supplied very largely by 2 per cent of milk fat from evaporated milk (28 cc. per 100 gm. of ration), and fresh milk (56 cc. per 100 gm. of ration), respectively, gave no evidence of the destruction of this vitamine (Chart 7). During the period of investigation both groups have made normal growth gains, and in neither has there been any indication of xerophthalmia. The purified ration for these particular groups was prepared from casein obtained from centrifuged milk. This was not ether-extracted, nor was the wheat embryo used as

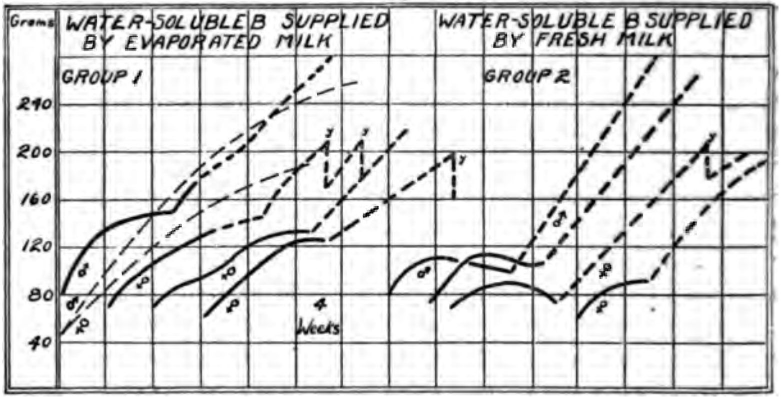


Chart 6. Animals fed purified rations in which evaporated milk (100 cc. during Period 2), Group 1, and fresh milk (200 cc. during Period 2), Group 2, respectively, supplied the only water-soluble vitamine made comparable growth gains.

the source of the water-soluble vitamine extracted previous to the alcoholic treatment. It is obvious, therefore, that somewhat more than 2 per cent of butter fat was being fed. But since it has been shown that 5 per cent is essential for normal growth,[12] it was believed that if a considerable destruction of the butter fat had taken place in the superheated milk it would be made apparent by the comparative study.

The addition of both vitamines—the water-soluble and the fat-soluble—produced no growth stimulation in the stunted animals fed the evaporated milk. If either of these vitamines was affected by the methods of heating, it was not indicated by our investigation.

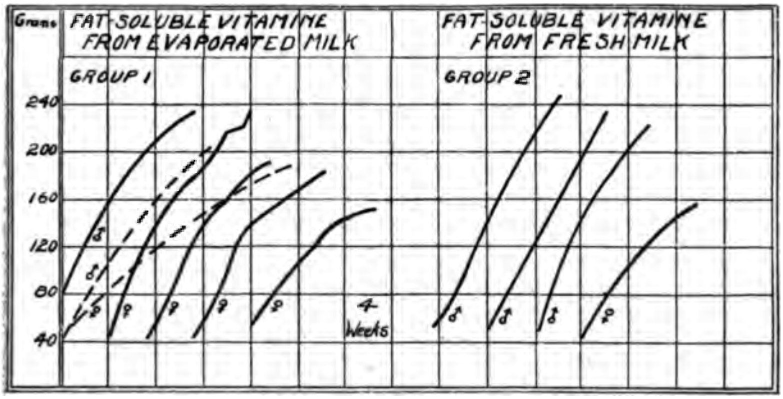


Chart 7. Growth curves of animals fed purified rations in which the fat-soluble vitamine was supplied by 2 per cent of fat from evaporated milk (Group 1), and fresh milk (Group 2). In these rations the casein and wheat embryo which furnished the extract containing the water-soluble vitamine were not ether-extracted.

The insoluble precipitate on the sides and in the bottom of some of the cans of evaporated milk used in the investigation suggested that the inorganic constituents, in part at least, might be responsible for our growth failures. It is well known that boiling brings about changed relations in the inorganic complexes of the milk, resulting in an increase of the insoluble calcium and magnesium salts, especially the calcium phosphates,[13] at the expense of the soluble forms. But all evidence in stock feeding, at least, points to the conclusion that these di- and tri-basic salts are available. Bone ash and precipitated calcium phosphate are usual additions to certain types of farm rations.[14] The additions of mono-basic and di-basic calcium phosphate, respectively, to our milk foods were without significant influence. The mono-basic salt was apparently distasteful, for when this was added very little of the milk was eaten. With the di-basic phosphate somewhat better results were obtained, but growth was still considerably below normal. When calcium lactate was added to the milk brought slowly to the boiling point, growth was stimulated in animals previously stunted on this milk alone (Chart 8). Furthermore, young animals fed both the long heat-treated milk, and the superheated milk (evaporated) to which calcium lactate (approximately 0.77 gm. per 100 cc. of milk) was added from the beginning, made fairly satisfactory growth gains; and in a number of cases young were born (Charts 9 and 10). Very occasionally we succeeded in raising a few of a second generation. However, the number of young which we failed to

---

[12] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641.

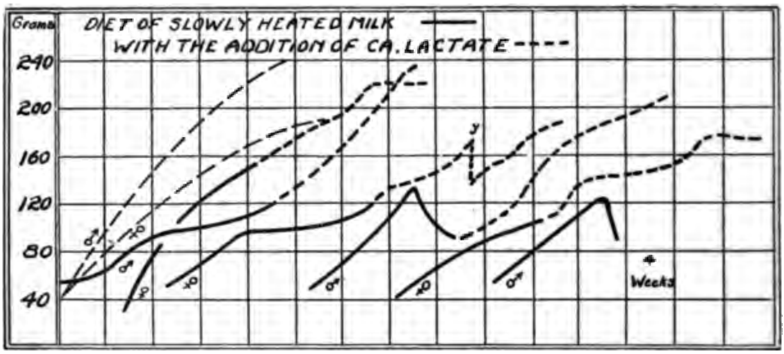


CHART 8. The addition of calcium lactate to milk brought slowly (35 minutes) to the boiling point, stimulated growth (Period 2). The rate of growth, however, is considerably less than the optimum.

---

[13] *Bull. Hyg. Lab., U. S. P. H. No. 56*, 1909, 2nd edition, 646.

[14] Henry, W. A., and Morrison, F. B., Feeds and feeding, Madison, 16th edition, 1916, 66.

raise, and the fact that our first generation was somewhat below the accepted standard for well nourished rats, suggested that calcium lactate only in part made up the deficiency produced by the heat treatment of milk. The additions of considerably larger amounts of calcium lactate produced no better results.

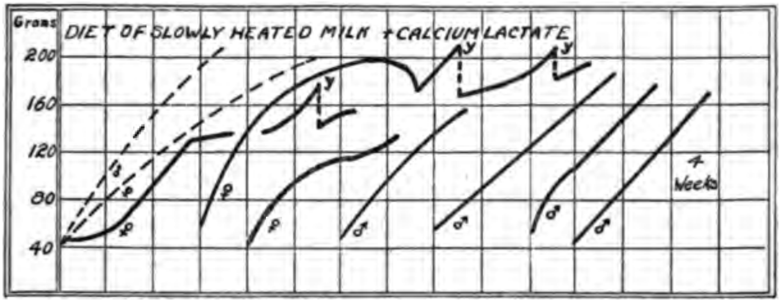


Chart 9. These animals were fed, from the beginning of the experimental period, milk brought slowly to the boiling temperature, to which calcium lactate was added.

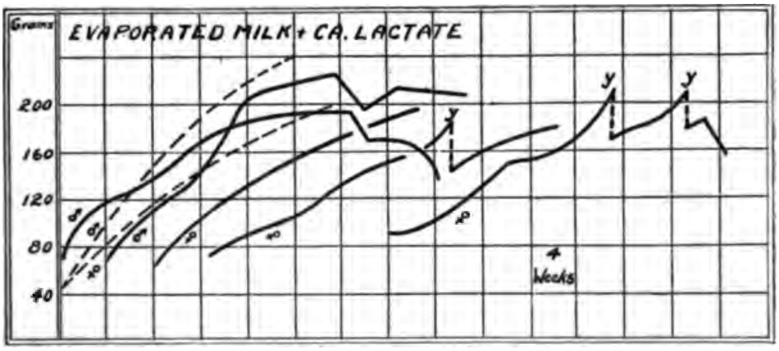


Chart 10. The addition of calcium lactate to evaporated milk produced much better growth than evaporated milk alone. In some cases young were born; a few lived through the suckling period.

It seemed probable that our lack of success with the dicalcium phosphate might have been due to the fact that enough of the salt could not be dissolved in the milk to meet the needs of the experimental animals, therefore the more soluble calcium glycerophosphate was tested. The substitution of this for the calcium lactate stimulated growth (Chart 11, Group 1). By incorporating the calcium glycerophosphate in a paste of diluted evaporated milk and corn-starch, our results were even more successful. The growth curves of these animals (Chart 11, Group 2) were quite similar to those of animals fed milk boiled 1 minute. With our calcium glycerophosphate additions young rats have grown to maturity and reproduced at a comparatively early age. This is the first time in our many experiments with long heat-treated, or superheated milk that we have been able to secure results at all comparable with those obtained on raw milk (Chart 12) or milk brought quickly to the boiling point (Chart 1).

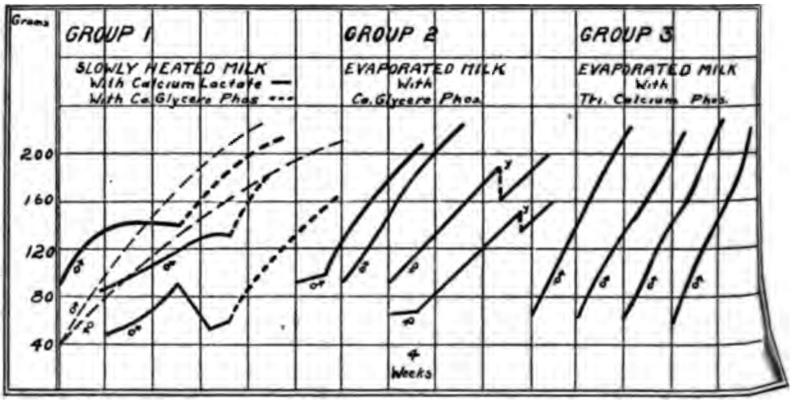


Chart 11. The substitution of calcium glycerophosphate for calcium lactate in the diet of rats fed slowly heated milk stimulated growth (Group 1).

Calcium glycerophosphate added to evaporated milk produced normal growth curves. Young were born at a comparatively early age (Group 2).

Evaporated milk supplemented with tricalcium phosphate in a starch paste produced equally good growth (Group 3).

As has been stated, all evidence in the literature is to the effect that tricalcium phosphate when fed to stock is available. Do rats differ from farm animals in being unable to utilize this tri-basic salt? In order to determine this we fed evaporated milk (Brand A), and supplemented this with the insoluble salt incorporated in a starch paste. The growth of these animals (Chart 11, Group 3) is superior to that of any of our milk-fed rats, and leaves no doubt concerning the availability of this tri-basic salt.

Why then did our animals fail to grow on the long heat-treated and evaporated milk? Two possibilities suggest themselves. In the process of condensing, a certain amount of the calcium phosphate is precipitated and may be discarded as waste, although in the published analyses[15] there is no evidence that such has been the case. It is also possible that some of the insoluble material settled to the bottom of the feeding containers and was not taken by the animals. Rats fed the diluted evaporated milk (Brand A) thickened with corn-starch made practically normal growth gains during the 8 weeks of the investigation (Chart 5, Group 1). A comparison of these curves with those of our animals on the unthickened evaporated milk suggests that the growth failures of our animals on the evaporated milk were due to the fact that the insoluble calcium phosphate in the diluted milk settled out and was not eaten.

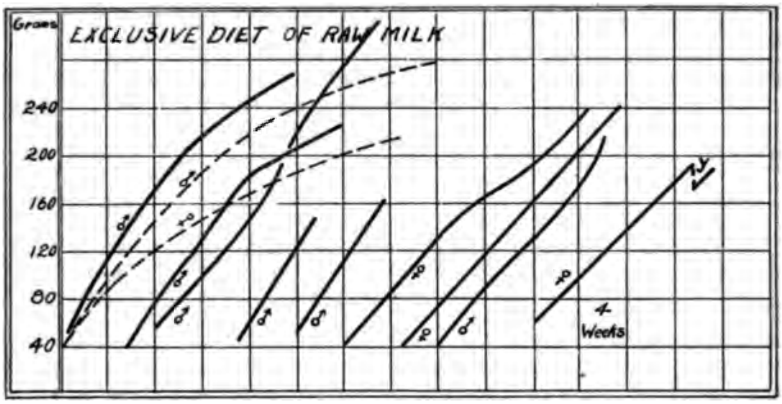


CHART 12. Growth curves of first generation rats fed raw milk.

Can the nutritive failures of our animals fed the long treated milk, that is the milk brought slowly to the boiling point, and the pasteurized milk, be similarly accounted for? In these we have never been conscious of any considerable precipitate, although slight precipitates were frequently observed on the sides and bottom of the containers in which the milks were heated. In order to test this point, animals previously stunted as the results of pasteurized milk feeding were given in addition the tricalcium phosphate starch paste. As in the case of the evaporated milk-fed rats growth was immediately stimulated (Chart 3), suggesting that in the "hold" process of pasteurization the insolubility of the calcium salts was responsible for the growth failures. When the bottles in which the milk was pasteurized were carefully washed out with distilled water and the washings incorporated in a starch paste, the animals made better gains than on the pasteurized milk alone (Chart 13, Group 2). Similarly, our animals fed long heat-treated milk incorporated into a paste made normal growth gains (Chart 13, Group 1). In both these cases the results can be explained only by the fact that the calcium phosphates were held in suspension by the colloidal solution and therefore were made available.

---

[15] Sherman, H. C., Chemistry of food and nutrition, New York, 2nd edition, 1918, 424.

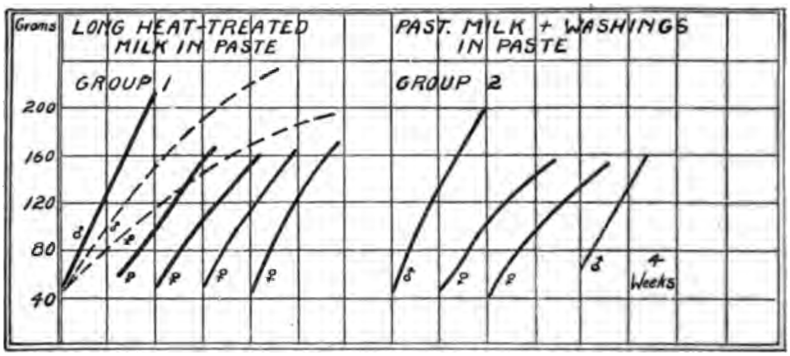


Chart 13. Long heat-treated milk made into a paste also produced growth superior to that of the long heat-treated milk alone (Group 1).

The washings from the containers in which the milk was pasteurized, when added to the pasteurized milk in the form of a paste, produced better growth than the pasteurized milk alone (Group 2).

The growth of the animals fed the thickened heat-treated milk suggested an explanation for our results with the condensed milk. This milk, it will be recalled, is of a thick, semisolid consistency, and in our experiments it happened that this was fed *undiluted*. Any insoluble calcium salts formed during the condensing process, therefore, were held in suspension. The thick mixture made it possible for the animals to get all the calcium phosphates present.

The explanation for the results obtained in the previous investigation[8] following the addition of egg yolk and casein to the long heat-treated milk, is now apparent. Both of these materials furnished appreciable amounts of calcium and phosphorus—substances which have been shown to be deficient in long heat-treated milk.

The results of all our experiments on the long heat-treated milks point to the same conclusion; namely, that, in the process of heating, the calcium salts are rendered more or less insoluble, depending upon the length of time the milk is heated. In this insoluble form they may be lost, owing to the fact that some of the precipitated material adheres to the container, as in the case of long pasteurized or slowly heated milk, while some, for example in evaporated milk, separates out on standing. When especial care was taken to include the insoluble material by colloidal suspension, results comparable to those on raw and quickly boiled milk were obtained. We have secured no data indicating that either the fat-soluble or the water-soluble vitamine in milk is affected by heat treatment. Nor is the casein apparently affected. Rats fed superheated milk supplemented with calcium phosphate properly incorporated made normal growth gains. The inferior growth of rats on the long heat-treated and superheated milk appears to be due wholly to the readjustments of the inorganic complexes.

The application of these findings to infant nutrition is now under investigation.

BIOCHEMICAL STUDIES ON MARINE ORGANISMS.

II. THE OCCURRENCE OF ZINC.

By MEYER BODANSKY.

(*From the Laboratory of Biological Chemistry of the School of Medicine, University of Texas, Galveston.*)

(Received for publication, August 25, 1920.)

Our knowledge concerning the normal occurrence of zinc in animal tissues has been very limited until quite recently. The earlier investigators demonstrated that traces of zinc might be found in toxicological analyses in the absence of poisoning by this metal. Raoult and Breton (1877) reported four analyses of human livers in which the zinc content varied between 10 and 76 mg. per kilo. Lechartier and Bellamy (1877) made similar analyses, and in addition reported the presence of this metal in the muscle and liver of an ox, and in wheat, corn, barley, and other grains. The researches of Javillier (1908, *a*) established the widespread occurrence of zinc in the plant kingdom.

More recently, the investigations of Weitzel (1914), Rost and Weitzel (1919), Delezenne (1919), and Rost (1920) have contributed toward a fuller appreciation of the significance of this problem. Birckner (1919) estimated the zinc content of a number of food products and, because of its constant occurrence in the yolk of eggs as well as in cow's and human milk, he was led to infer that the element zinc exerts an important nutritive function.

Marine animals are of especial interest in this respect. Bradley (1904) and Mendel and Bradley (1905) discovered zinc to be a normal constituent of the liver and the blood of the mollusks *Sycotypus canaliculatus* and *Fulgar carica*. Various other marine animals, including some of the most common mollusks and Crustacea of the Long Island coast, were examined for zinc, but, with the exception of *Urosalpinx cinerea*, no appreciable amounts of the element could be found. Mendel and Bradley expressed their belief, however, that other marine forms may contain this element in their tissues. Phillips (1917) analyzed the mineral content of the tissues of certain Tortugas marine organisms and found zinc in all except the crayfish blood. He postulated that zinc must be considered as a normal constituent in these forms since the possibility of contamination of the sea water was excluded. Recently Hiltner and Wichmann (1919) determined the zinc content of oysters from various sources and on the basis of their results concluded that zinc is present universally in oysters, or, at any rate, in those grown in Atlantic waters along the coast of the United States.

The indications are that zinc is a physiological constituent of animal and vegetable tissue and its presence in large quantities in some invertebrates may signify that it plays an important rôle in these forms. In view of these considerations, and because of the paucity of information on this subject, it was thought desirable to investigate the zinc content of a fairly large number of marine animals, including the higher fishes, and to determine, if possible, the distribution of this metal in their tissues.

EXPERIMENTAL.

In the determination of zinc the filtrates from the copper estimations, previously reported by Rose and Bodansky (1920), were used. The zinc was precipitated as the sulfide in a solution acid with citric acid. The turbidimetric procedure employed by Birckner (1919) was followed. The reagents used in this work were carefully tested for zinc. That no contamination resulted from the glassware and other apparatus was ascertained by making several blank determinations. In no instance was zinc detected.

The sensitiveness and limitations of the turbidimetric comparison are amply discussed by Birckner (1919). Since a slight error in reading turbidities is inherent in the method, and since frequently in our work only a small portion of the total volume was taken for comparison, there is the possibility of an error being multiplied many times.

In Table I are recorded comparative data for the turbidimetric method and the gravimetric procedure as employed by Jamieson (1919) for the estimation of zinc in gelatin. The results obtained demonstrate the applicability of the method to the estimation of such quantities of zinc as were encountered in our work. It should be stated that the turbidities produced by 0.2 to 0.3 mg. of zinc can be compared with the greatest degree of accuracy.

Five samples of oysters and one of clams obtained at different times and from different sources were all found to contain zinc. The amount present in the oysters varied between 188.5 and 341.0 mg. per kilo, the average being 259.9 mg. (Table II). These results are in accord with the findings of Hiltner and Wichmann (1919) and serve to establish the occurrence of zinc not only in oysters grown in Atlantic waters, but in those grown in the Gulf of Mexico as well. It is probably safe to assume that zinc is an ever occurring constituent of the oyster.

TABLE I.

Comparison of the Turbidimetric and Gravimetric Methods.

Sample No.	Zinc in sample.	Zinc found.	
		Gravimetric method.	Turbidimetric method.
	mg.	*mg.*	*mg.*
1	0.10		0.12
2	0.30		0.28
3	0.50		0.49
4	1.00	0.76	0.96
5	2.50	2.56	2.50
6	5.00	5.06	5.20
7	10.00	9.69	9.75
8	15.00	15.64	15.50
9	20.00	20.34	20.50

There is indeed very little information concerning the zinc content of sea water. Dieulafait (1880) reports that the waters of the Mediterranean normally contain at least 2 mg. of zinc in a cubic meter. Hiltner and Wichmann (1919) report an analysis of a sample of sea water taken from Great South Bay, 1 mile off shore from West Sayville, New York, in which no zinc was detectable in 3,500 cc. A sample of water from the Gulf of Mexico taken about 30 feet from the Galveston shore contained 0.0073 mg. per kilo. This figure is considerably higher than that

TABLE II.

Zinc Content of Marine Animals.

No. of analysis.	Animal.	Amount taken for analysis.	Zinc.		Remarks.
			Found in sample.	In 1,000 gm. of fresh substance.	
		gm.	*mg.*	*mg.*	
	Cœlenterata.				
9	Jellyfish.	200.0	2.50	12.50	Entire animal ground.
42	Portuguese man-of-war.	271.5	1.22	4.50	One animal entire.
44	" "	81.0	1.30	16.05	Two small individuals.
	Pelecypoda.				
2	Oysters.	100.0	18.85	188.50	Shucked.
4	"	100.0	34.10	341.00	
31	"	100.0	27.50	275.00	
32	"	100.0	24.38	243.80	
37	"	100.0	25.11	251.10	
39	Clams.	76.6	5.90	77.00	
	Crustacea.				
3	Shrimps.	100.0	1.73	17.30	Includes protective covering.
70	"	193.5	3.25	16.80	
5	Crabs.	100.0	2.28	22.80	
10	"	100.0	1.83	18.30	
	Elasmobranchii.				
29	Torpedo ray.	100.0	0.33	3.30	Three small fish ground and sampled.
16	Sting ray.	100.0	0.39	3.90	Entire animal ground and sampled.
48	" "	160.3	0.60	3.74	One animal entire.
	Teleostomi.				
6	Mullet.	100.0	0.25	2.50	One or more individuals thoroughly ground and mixed before sampling.
15	"	100.0	0.28	2.80	
17	Redfish.	100.0	0.28	2.80	
21	Drumfish (young).	86.0	0.48	5.58	
14	Croaker.	100.0	0.64	6.40	
25	Whiting.	100.0	0.74	7.40	
30	Tarpon.	100.0	0.76	7.60	
19	Angelfish.	59.0	0.46	7.80	
23	Pigfish.	43.3	0.36	8.40	
28	Yellow-tail.	94.0	0.80	8.50	
22	Drumfish (older than No. 21).	100.0	0.96	9.60	
18	Spotted trout.	100.0	1.02	10.20	
12	Flounder.	100.0	1.16	11.60	
27	"	100.0	1.69	16.90	
24	Sea-catfish.	100.0	9.75	97.50	
11	"	100.0	12.70	127.00	
47	"	139.0	20.73	149.10	One animal entire.

given by Dieulafait, and although there is no obvious contamination due to industrial wastes, the writer hesitates to present this as being the normal value for sea water. It may, however, be taken as the maximum for the water in which these animals live.

It is therefore a striking phenomenon that the oyster living in a water, not contaminated by industrial wastes, should contain in its tissues more than 35,000 times as much zinc as is present in an equivalent weight of water. Apparently the oyster is capable of absorbing and retaining the zinc which it obtains to a slight extent perhaps from the water, but mainly from its diet of diatoms, Infusoria, minute Crustacea, etc.

A dialysis experiment was undertaken to determine the nature of the combination of zinc with the tissues of the oyster. Finely ground oysters weighing 87.5 gm. and containing 21.97 mg. of zinc were dialyzed through a parchment bag for 96 hours under aseptic conditions. The water was changed after the first 26 hours. 10.87 mg. or 49.5 per cent of the zinc were recovered in the dialysate. The significance of this result becomes apparent when it is contrasted with the findings for copper obtained under similar conditions by Rose and Bodansky (1920). In the copper experiment 0.5 mg. or 12.2 per cent of the total Cu appeared in the dialysate. The zinc may be present far in excess of its combining power with the proteins of the tissue, or it may exist in part in a loosely combined state which is disintegrated during dialysis.

In order to determine the distribution of zinc in the oyster, 525 gm. were introduced into 10 per cent formaldehyde solution for 24 hours and dissected into four fractions: (*a*) digestive glands or hepatopancreases, (*b*) mantles, (*c*) adductor muscles, and (*d*) other parts including the gills which we have called the "residue." The formaldehyde solution was analyzed separately. This experiment was later repeated on 300 gm. of oysters without the formaldehyde treatment. The results are summarized in Tables III and IV. As will be observed the zinc is distributed rather evenly in the digestive organ, mantle, and gills. The zinc content of the muscle is considerably lower than that of the other tissues.

TABLE III.

Distribution of Zinc in Oysters, Hardened in 10 Per cent Formaldehyde for 24 Hrs. Previous to Dissection.

Fraction.	Weight.	Zinc.	
		Found in sample.	In 1,000 gm. tissue.
	gm.	*mg.*	*mg.*
Digestive glands	80.0	19.00	237.50
Mantles	141.7	37.84	267.00
Muscle	70.3	11.25	160.00
"Residue"	206.0	55.50	269.40
Formaldehyde extract		6.50	
Total	498.0*	130.09†	

* 525 gm. of oysters were dissected. The difference of 27 gm. represents the water which passed into the formaldehyde and that which was lost during the dissection.

† Calculated on the basis of 525 gm., this is equivalent to 247.8 mg. of zinc per kilo.

TABLE IV.

Distribution of Zinc in Oysters, Dissected without Previous Formaldehyde Treatment.

Fraction.	Weight.	Zinc.	
		Found in sample.	In 1,000 gm. tissue.
	gm.	*mg.*	*mg.*
Digestive glands	56.7	11.10	196.00
Mantles	84.0	18.38	218.80
Muscle	38.5	2.94	76.40
"Residue"	102.0	19.50	191.20
Total	281.2*	51.92†	

* 300 gm. of oysters were dissected. The difference of 18.8 gm. represents the water which was lost during the dissection.

† Calculated on the basis of 300 gm., this is equivalent to 173.1 mg. of zinc per kilo.

The two cœlenterates examined, the jellyfish, *Aurelia*, and the Portuguese man-of-war, *Physalia*, contained from 4.50 to 16.05 mg. of zinc per kilo of moist tissue.

Analyses were made of two species of Crustacea, the crab and the shrimp. The meats and hulls of 200 gm. of shrimps were analyzed separately. The meats weighing 104.5 gm. contained 1.40 mg. of zinc; the hulls weighing 89.0 gm. contained 1.85 mg. of zinc. In terms of mg. per kilo of tissue, the meats and hulls had 13.5 and 20.8, respectively. 6.5 gm. of water were lost in separating the hulls from the meats.

Fourteen species of fish including two elasmobranchs, the torpedo ray and the sting ray, were analyzed. Zinc was found in all, and the amount varied between 2.5 mg. per kilo in the mullet and 149.1 mg. in the catfish. The catfish holds a unique position among the teleosts examined, in that its content of zinc is entirely out of proportion to that found in any other species. In an extensive research, Delezenne (1919) established the occurrence of large quantities of zinc in the venom of snakes. The catfishes *Siluridæ* possess poison glands opening on the spine of the pectoral fin (Parker and Haswell, 1897). In preliminary experiments to determine the rôle, if any, of zinc in the catfish, the possibility of the high zinc content being in some way associated with the poison glands was not overlooked. The fins including the poison glands of two small catfish were analyzed separately from the remaining tissue. The fins, weighing 14 gm. contained only 0.0667 mg. of zinc, or 4.76 per kilo. The remaining tissue, weighing 125 gm., contained 20.67 mg. of zinc, or 165.36 mg. per kilo. No information concerning the rôle of zinc in the catfish is adduced from this experiment.

Whether the presence of comparable quantities of zinc is characteristic of other species of catfish remains to be determined. Although there is the possibility that in this species zinc may have a special and yet undetermined function, it is probable that the high zinc content is due to the peculiar feeding habits of the animal. Investigations have shown that mollusks constitute an important article of the food for the catfish (Kendall, 1910). Attention should be called to the fact that, whereas the catfish is high in zinc, only traces of copper have been detected in the individuals thus far examined by Rose and Bodansky (1920).

The constant occurrence of zinc in every species analyzed indicates that it is a physiological constituent of the tissues of marine animals. With the exception of the catfish, the amounts vary between 2.5 and 16.9 mg. per kilo in the teleosts, being considerably higher than the figures previously reported for copper. The presence of zinc in these proportions is not limited, however, to marine animals, for various articles of food contain comparable quantities of this element, and much larger amounts may be present in the tissues of terrestrial vertebrates (Rost, 1920). Mendel and Bradley (1906–07) showed that zinc in *Sycotypus canaliculatus* is an essential constituent of the respiratory protein which they named "hemosycotypin," and which is analagous to the hemocyanins. In the oyster and the clam zinc may possibly have a similar function. In other marine animals, however, the biological significance of zinc is less apparent or entirely obscure.

The favorable stimulatory influence of minute quantities of zinc on the growth of *Aspergillus niger* was observed by Raulin (1870). Javillier (1907, 1908) confirmed and extended Raulin's work and demonstrated (1912) that in the growth of *Sterigmatocystis nigra* no other element could replace zinc. Cadmium alone produced measurable stimulatory effects, and since this element bears many chemical resemblances to zinc, Javillier justly concludes that the physiological action of zinc is specific. The specificity and similarity in behavior of Zn and Cd ions on the enzymatic activity of yeast have been reported by Kostychev and Zubkova (1916). Delezenne (1919) also associates zinc with the activity of enzymes. Significant as these results are, no generalizations can be made concerning the rôle of zinc in the tissues of marine vertebrates until more information becomes available.

SUMMARY.

1. Zinc has been found and quantitatively determined in twenty species of marine animals. The constancy of its occurrence indicates that it is a normal constituent of the tissues of these forms.

2. In the oyster zinc is distributed rather uniformly in the digestive organ, mantle, and gills, but the muscle contains less zinc than do the other tissues.

3. On dialysis of finely ground oysters for 96 hours, 49.5 per cent of the zinc was recovered in the dialysate. The zinc may be present in excess of its combining power with the proteins of the

tissue, or it may exist in part in a loosely combined state which is disintegrated during dialysis.

In conclusion the writer wishes to express his indebtedness to Professor William C. Rose of this Laboratory at whose suggestion and under whose guidance this work was done.

BIBLIOGRAPHY.

Birckner, V., *J. Biol. Chem.*, 1919, xxxviii, 191.

Bradley, H. C., *Science*, 1904, xix, 196.

Delezenne, C., *Ann. Inst. Pasteur*, 1919, xxxiii, 68.

Dieulafait, L., *Compt. rend. Acad.*, 1880, xc, 1573.

Hiltner, R. S., and Wichmann, H. J., *J. Biol. Chem.*, 1919, xxxviii, 205.

Jamieson, G. S., *J. Ind. and Eng. Chem.*, 1919, xi, 323.

Javillier, M., (*a*) Thesis, Paris, 1908; *Compt. rend. Acad.*, 1907, cxlv, 1212; (*b*) 1908, cxlvi, 365; 1912, clv, 1551.

Kendall, W. C., *U. S. Dept. Com. and Labor, Bureau of Fisheries, Document 733*, 1910, 12.

Kostychev, S., and Zubkova, *J. Russ. Bot. Soc.*, 1916, i, 47; *Physiol. Abstr.*, 1919, iv, 416.

Lechartier, G., and Bellamy, F., *Compt. rend. Acad.*, 1877, lxxxiv, 687.

Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1905, xiv, 313; 1906–07, xvii, 167.

Parker, T. J., and Haswell, W. A., Text book of zoology, London, 1897, ii, 212.

Phillips, A. H., *Carnegie Inst. Washington, Pub. 251*, 1917, xi, 91.

Raoult, F., and Breton, H., *Compt. rend. Acad.*, 1877, lxxxv, 40.

Raulin, J., Thesis, Paris, 1870.

Rose, W. C., and Bodansky, M., *J. Biol. Chem.*, 1920, xliv, 99.

Rost, E., *Umschau*, 1920, xxiv, 201.

Rost, E., and Weitzel, A., *Arb. Reichsgesheitsamt*, 1919, li, 491.

Weitzel, A., *Zentr. Physiol.*, 1914, xxviii, 766.

ESTIMATION OF THE PHENOLIC SUBSTANCES IN URINE.

By FREDERICK F. TISDALL.

(*From the Department of Pediatrics, Johns Hopkins University, Baltimore.*)

(Received for publication, August 19, 1920.)

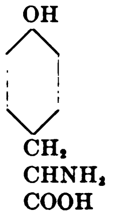
In the present paper is presented a review of the literature on which is founded our knowledge of the phenolic substances in the urine, a survey of the various methods for determining the amount of volatile phenols in the urine, and an investigation of Folin and Denis' colorimetric method for the estimation of the total phenolic substances (1).

There is some confusion in the literature as to the meaning conveyed by the word phenol. In the older literature the word was used as meaning volatile phenols, but since the appearance of Folin and Denis' method it has been largely used to designate all the phenolic substances. These include volatile and non-volatile phenols and aromatic oxy-acids. The former are a group of compounds characterized by a benzene ring in which one or more of the hydrogen atoms have been replaced by a hydroxyl group, to which they owe their specific character. The latter, the aromatic oxy-acids, have a carboxyl group in addition to the benzene ring and free hydroxyl group. In this paper the term phenol will be restricted to the compound having the formula C_6H_5OH. The aromatic oxy-acids usually found in urine are *p*-oxyphenylpropionic acid (hydro-*p*-cumaric acid), *p*-oxyphenylacetic acid, and *p*-oxybenzoic acid; the volatile phenols are *p*-cresol and phenol; and there may be normally present one non-volatile phenol, pyrocatechin. All these substances have their origin from the amino-acid tyrosine.[1]

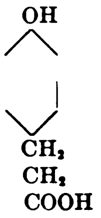
---

[1] From the chemical standpoint tyrosine might very properly be regarded as belonging to the phenolic group of substances, but from the metabolic standpoint it is preferable that it should not be included in this group. There may also be other compounds in the urine which are pro-

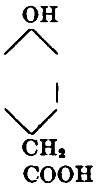
Tyrosine.



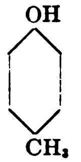
p-Oxyphenylpropionic acid.



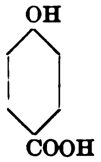
p-Oxyphenylacetic acid.



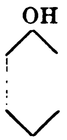
p-Cresol.



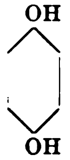
p-Oxybenzoic acid.



Phenol.



Pyrocatechin.



Volatile Phenols.

Phenol was first discovered in horse, cow, and human urine by Städeler (3) in 1851.[2] He identified phenol and a substance which he called "Taurylsaure" which was later shown to be

duced during the metabolism of tyrosine by the body, which could properly be included as phenolic compounds. This is certainly the case in the abnormal condition of alkaptonuria, in which homogentisic acid is produced. These compounds have been regarded by most writers from the metabolic standpoint and not considered as phenolic bodies. Folin and Denis (2) in a brief review of the literature state "Weyl . . . . succeeded in showing that putrefactive bacteria . . . . produce large quantities of phenols from tyrosine," and also in considering the phenolic substances in urine they say "The most important phenol quantitatively is in fact paracresol. In addition there is in urine some orthocresol and at least two dioxyl benzenes namely pyrocatechin and hydroquinone as well as some aromatic oxy acids, particularly paraoxybenzoic acid."

[2] "Ganz besonders uberraschend war aber das Auftreten der Phenylsaure (Carbolsaure) die ich nicht allein im Harn der Herbivoren sondern auch in Menschenharn aufgefunden habe."

p-cresol. In 1867 Schultzen and Naunyn (4) demonstrated the presence of phenol in human urine after giving large doses of benzene but were unable to show its presence in normal urine. Although Städeler had shown in 1851 that phenol was present in normal human urine, the credit for this discovery has been generally given to Landolt (5) who in 1871 proved its presence, using a comparatively small quantity of urine.

The volatile phenols in human urine were shown by Brieger (6) to be largely *p*-cresol and a small amount of phenol. *o*-Cresol was not isolated directly but its presence was inferred from the finding of a small quantity of salicylic acid which is an oxidation product of *o*-cresol. The occurrence of *o*-cresol in human urine is very doubtful as Baumann (7) proved that the above evidence was not conclusive.

Städeler obtained phenol from urine by distilling with sulfuric acid and concluded that it was present in the free state. Buliginsky (8) found no free volatile phenol on distilling fresh horse urine, but obtained a large quantity on distilling after the addition of a dilute mineral acid. Hoppe-Seyler (9) confirmed Buliginsky's findings and hence it was concluded that there was a phenol-forming substance in the urine which liberated the volatile phenols on heating with mineral acids.

Further knowledge of the phenol-forming substance was not obtained until 1876. In 1876 Baumann (10) showed that after precipitating all the sulfates in the urine a second amount was obtained by heating the urine with concentrated HCl. He designated these sulfates as "paired sulfuric acid" (ethereal sulfates). He (11) noted that the urine of patients who had been exposed to carbolic acid gave little or no volatile phenols on simple distillation but a large quantity was obtained on distilling after the addition of HCl. At the same time he found a marked increase in the ethereal sulfates and concluded from this that the volatile phenols were bound with sulfuric acid. He verified his conclusions by isolating from the urine the potassium salt of the phenol sulfuric ester.

Baumann also investigated the origin of the phenol sulfuric ester in the body (11). He obtained from the liver of a dog which had been poisoned with carbolic acid nineteen times more phenol than in a similar quantity of blood. He also observed that the quantity of sulfates in the liver was not sufficient to have united with all the phenol found and concluded that there was a second phenol-forming subtance. This was later shown by Schmiedeberg (12) to be the ester phenol glucuronic acid.

The source of the volatile phenols was not known for a long time. It was assumed on account of their abundance in the urine of herbiverous animals that they arose from plant food. Schultzen and Naunyn (4) and later Munk (13) had also demonstrated that phenol could be formed in the body by the oxidation of benzene. Baumann (14) inferred from the observation that volatile phenols were found in the urine of animals which had been fed on an exclusive meat diet that they did not have their origin from plant food but from protein. He then demonstrated the formation of a considerable quantity of phenol in the putrefaction of protein with pancreas and also made the interesting observation that phenol did not occur in any quantity until about the 6th day. He assumed from this that the phenol was not formed directly from protein but was a secondary or further decomposition product. It was later shown by Baumann and Brieger (15) that the volatile phenols formed by the putrefaction of protein were largely *p*-cresol and a small quantity of phenol. The presence of *o*-cresol was inferred from the finding of a small quantity of salicylic acid. Shortly after this Weyl (16) demonstrated the formation of *p*-cresol and phenol in the direct putrefaction of tyrosine. Brieger (17) had already found volatile phenols in feces and intestinal contents.

A further survey of the literature shows very clearly that none or only a trace of the volatile phenols is produced by the body, outside of the intestinal canal, except in certain diseases such as empyema, erysipelas, pyemia, putrid bronchitis, gangrene, etc. (18, 19). A discussion of this aspect of phenol metabolism will not be gone into beyond calling attention to the experiment of Baumann (20) in which he gave a fasting dog calomel and found that the volatile phenols disappeared from the urine; and also to a case, which he records (20), in which the urine of a patient who was suffering from an intestinal fistula showed only a trace of volatile phenols. In a similar case Ewald (21) found no volatile phenols in the urine but they appeared again after the fistula was closed.

The evidence is thus complete that volatile phenols in human urine have their origin in the putrefaction of tyrosine in the intestinal canal. They are then absorbed and conjugated in the body with sulfuric and glucuronic acid to be excreted in the urine as conjugated volatile phenols.

Non-Volatile Phenols.

Pyrocatechin.—The only non-volatile phenol that has been found in normal human urine is pyrocatechin (brenzcatechin). Ebstein and Müller (22) first demonstrated its presence in the urine of a healthy child in 1875. Baumann (23) showed that it was a constant constituent of horse urine. He also found a small quantity, the amount varying from day to day, in the urine of a healthy 12 year boy (23) and from this and other observations concluded that pyrocatechin was a frequent, if not a normal, constituent of human urine.

Baumann and Preusse (24) demonstrated that pyrocatechin was formed by the oxidation of phenol, and after giving a dog large quantities of phenol found that the pyrocatechin in the urine was excreted entirely in the conjugated state. The formation of pyrocatechin from phenol is thus seen to be analogous to the oxidation of benzene to phenol. It is of interest to note that Nencki and Giacosa (25) were able to demonstrate the presence of both phenol and pyrocatechin in the urine of a man who was receiving daily 6 gm. of benzene.

Hydroquinone.—Hydroquinone has not been found in normal human urine. Nencki and Giacosa were able to show the presence of a small quantity in the urine of the above mentioned case. Baumann and Preusse (24) obtained over 1 gm. of hydroquinone in 6 days from the urine of a dog, to which phenol was given, it being present entirely in the conjugated state.

Aromatic Oxy-Acids.

p-Oxyphenylpropionic Acid.—Baumann observed that in the putrefaction of protein phenol did not appear until about the 6th day and concluded from this that it was not formed directly from protein but through intermediate products. At the end of 2 days in the putrefaction of tyrosine he obtained by ether extraction a substance which he identified as *p*-oxyphenylpropionic acid (26). As an evidence of the amount of *p*-oxyphenylpropionic acid that could be obtained in this manner he isolated 12 gm. of the pure acid from 20 gm. of tyrosine (27). He also obtained the pure crystals from 0.5 liter of pus from a case of peritonitis (28).

The fate of *p*-oxyphenylpropionic acid in the body was investigated (27). 5 gm. of pure acid were given to a man and the urine for the following day was found to contain an increased amount of volatile phenols and about 0.8 gm. of the *p*-oxyphenylpropionic acid. It was thus shown that on feeding *p*-oxyphenylpropionic acid a large part disappeared in the organism, some was excreted unchanged, and a smaller part was excreted as volatile phenols. Its presence in normal urine was demonstrated by Baumann (28) when he isolated the pure crystals from 240 liters of urine.

p-Oxyphenylacetic Acid.—*p*-Oxyphenylacetic acid was found in the putrefactive products of serum albumin by Salkowski (29). It was first found in normal urine by Baumann (27) and later he isolated from the above mentioned 240 liters of urine 2 gm. of the pure acid. As Baumann did not find *p*-oxyphenylacetic acid in demonstrable quantities after feeding *p*-oxyphenylpropionic acid (27) he concluded that it was probably formed directly from tyrosine by splitting off CO_2 and NH_3. However, shortly after this he showed the putrefactive products of *p*-oxyphenylpropionic acid to be *p*-oxyphenylacetic acid, *p*-cresol, and phenol (28) and concluded that *p*-oxyphenylacetic acid could originate from both tyrosine and the *p*-oxyphenylpropionic acid. Weyl (30) demonstrated that the putrefaction of *p*-oxyphenylacetic acid in turn produced *p*-cresol.

p-Oxybenzoic Acid.—*p*-Oxybenzoic acid was shown by Baumann (7) to be formed from *p*-cresol. A dog was given 1 to 2 gm. of *p*-cresol daily, and from the urine pure crystals of *p*-oxybenzoic acid were obtained. After feeding 12 gm. of *p*-cresol, 1 gm. of pure *p*-oxybenzoic acid was isolated. The administration of 4 gm. of *p*-oxybenzoic acid produced a considerable increase in the amount of phenol in the urine. The direct production of phenol and CO_2 in the putrefaction of *p*-oxybenzoic acid had already been shown by Baumann (14).

Oxymandelic Acid and Gallic Acid.—There are two other aromatic oxy-acids that should be mentioned, oxymandelic acid and gallic acid. Schultzen and Riess (31) first demonstrated the presence of the former acid along with leucine and tyrosine in the urine of cases of acute yellow atrophy. It has not been found in normal urine. Gallic acid is sometimes found in considerable quantities in horse urine and is assumed to be a constituent of the food and not produced in the body (32). Its occurrence in human urine is doubtful.

By the above work it has been proved that *p*-oxyphenylpropionic acid originates from tyrosine and in turn forms *p*-oxyphenylacetic acid; *p*-oxyphenylacetic acid originates from *p*-phenylpropionic acid or directly from tyrosine and in turn produces *p*-cresol; and *p*-oxybenzoic acid is an intermediate product in formation of phenol from *p*-cresol.

The aromatic oxy-acids differ in two important respects from the volatile and non-volatile phenols. The first is that they do not produce any toxic effects even in large doses. This has been repeatedly shown throughout Baumann's experiments. The second is that they are largely excreted in the free state (28) while the volatile and non-volatile phenols have been shown to occur almost entirely in the conjugated form.

Methods for Estimating the Volatile Phenols.

Before 1871 no quantitative estimations of the volatile phenols were made. Their presence was demonstrated either by actually isolating them or by obtaining a dark violet color with phenol and a blue color with *p*-cresol, on the addition of ferric chloride. In 1871 Landolt (5) estimated the volatile phenols gravimetrically as tribromophenol. The earliest recorded figures by this method for the quantity of volatile phenols in human urine are those given by Munk (33). He found from 17 to 51 mg. per day.

Koppeschaar (34) in 1876 devised the first titration method which consisted in adding to the mixture to be estimated a known excess of bromic acid and alkaline bromide which on the addition of HCl furnished a definite amount of free bromine. This united with phenol to form tribromophenol (or tribromophenolbrom). The amount of bromine used was determined by adding potassium iodide, the potassium uniting with the remaining free bromine and liberating iodine which was titrated with thiosulfate. This method was shown by Kossler and Penny (35) to be very inaccurate for the estimation of *p*-cresol, the bromine taking considerable time to unite with the *p*-cresol and then gradually going on to form a tetrabromocresol. On account of this defect the method was of little use in estimating the volatile phenols in urine.

Messinger and Vortmann (36) in 1889 devised an iodine titration method which has remained the basis of all future titration methods. The procedure is as follows: An alkaline phenol (or *p*-cresol) solution is warmed to about 60° and to it is added a known amount of 0.1 N iodine solution. The iodine unites with the phenol to form triiodophenol. The solution is then cooled, acidified with dilute H_2SO_4, and the excess of iodine titrated with 0.1 N sodium thiosulfate.

In 1893 Kossler and Penny (35) using Messinger and Vortmann's principle of iodine titration devised a method for estimating the volatile phenols in urine. As acetone reacts with iodine it was necessary to remove it and this was accomplished by making the urine slightly alkaline and evaporating to one-fifth the original volume. All former investigations had shown that the volatile phenols occurred in the conjugated form so H_2SO_4 was added and the mixture heated, with repeated additions of water, until all the volatile phenols were distilled over. Other interfering substances in the distillate, such as ammonia, formic acid, and nitrous acid, were removed by the addition of calcium carbonate, and the volatile phenols again distilled. This second distillate was made alkaline with 10 per cent NaOH warmed to 60° and on cooling the excess of iodine was titrated with sodium thiosulfate. The amount of volatile phenols found by this method in normal human urine was from 70 to 106 mg. per day.

After being used for some years it was found that Kossler and Penny's method gave exceedingly high results with urine from diabetics. The reason for this was investigated by Neuberg (37) who showed that the high results obtained by Kossler and Penny's method were due to the formation of ketone or aldehyde bodies formed in heating the urine containing sugar with H_2SO_4. He overcame this difficulty by precipitating the volatile phenols in the first distillate as lead phenolate (and lead cresolate). The mixture was then heated and the interfering substances which were volatile escaped. Dilute H_2SO_4 was added to the basic lead phenolate and the second distillation and titration were carried out according to Kossler and Penny's method. In three cases the amounts of volatile phenols found by this method in human urine were 32, 35, and 32 mg. per day.

In 1909 Mooser (38) demonstrated that the distillation of urine with H_2SO_4 as carried out in the former methods, while not affecting phenol, destroyed from 20 to 30 per cent of the *p*-cresol present. He overcame this difficulty by using in place of H_2SO_4 an equal amount of 85 per cent phosphoric acid which did not destroy the *p*-cresol. Phosphoric acid also had the added advantage of not forming any interfering bodies with substances present in the urine and this obviated the necessity of precipitating the volatile phenols as lead phenolate. Mooser also found that during the second distillation with calcium carbonate some of both the phenol and *p*-cresol were lost. He overcame this difficulty by passing CO_2 through the solution. With these modifications the original Kossler and Penny method was carried out and correct values were obtained on known solutions of *p*-cresol and phenol. The amounts of volatile phenols found by Mooser in the urine of normal adults were 53, 39, 48, and 36 mg. per day.

The estimation of the volatile phenols has thus been shown to be fraught with difficulties. These difficulties were thought to have been overcome by the method as outlined by Mooser but the following year Neuberg and Hildesheimer (39) claimed that the distillation of urine, containing glucose or other carbohydrates, with 85 per cent phosphoric acid did form interfering substances. Thus the question of the estimation of volatile phenols was again in an unsettled state.

In 1912 Hensel (40) repeated Neuberg and Hildesheimer's experiments of distilling, with phosphoric acid, solutions containing glucose and *l*-arabinose, and obtained no iodine-binding substance. An ether extraction method was devised which consisted in extracting the volatile phenols from the first distillate by shaking with ether and then extracting from the ether with NaOH. Iodine was added to this solution and the excess titrated. The results recorded by Hensel were similar to those found by Mooser.

A colorimetric method was devised in 1898 by Bordas and Robin (41), which depended on the conversion of phenol into ammonium picrate. The color developed was compared with that given by a known amount of phenol. The standard was prepared by adding 5 mg. of phenol to 10 cc. of water. To this 1 cc. of a saturated solution of potassium nitrate and 2 cc. of concentrated H_2SO_4 were added. The solution was warmed on the water bath for 10 minutes, then saturated with NH_3, and diluted to 200 cc. From 1 to 10 cc. of this were taken and diluted to 50 cc., depending on the amount of phenol present in the unknown solution. The estimation of the volatile phenols was made by distilling the urine with H_2SO_4 until it was half its original volume. A measured portion of the distillate was taken and treated in the manner outlined for the standard solution. The color produced was compared to that produced by the known amount of phenol.

Another colorimetric method is also described by the same authors but they do not give any results obtained by either method.

Folin and Denis' Colorimetric Method.

This method is essentially different from the former methods in that it estimates not only the volatile phenols but also the non-toxic aromatic oxy-acids and any non-volatile phenols that may be present in the urine. Accordingly it is of little value to compare the results obtained, which are supposed to be a measure of the total phenolic substances with the figures recorded in the literature for the volatile phenols.

In 1912 Folin and Denis (42) described their phosphotungstic-phosphomolybdic reagent, the addition of which to a solution containing phenol in the presence of sodium carbonate develops an intense blue color. What the chemical combination is which produces this color has not been explained. The volatile phenols are not the only substances which cause the reaction as the blue color is also developed by the non-volatile phenols, the aromatic oxy-acids, tyrosine, protein, uric acid, and, as more recently shown by Folin and Wu (43), cuprous oxide. In the case of the phenolic substances the color is apparently due to the free hydroxyl group as no color is developed with the conjugated phenolic substances in which this group is united with sulfuric or glucuronic acid. On heating with HCl this union is broken down and the liberated phenolic substance will, of course, give the characteristic reaction.

Using this "phenol reagent" Folin and Denis devised their colorimetric method for the determination of the "total phenolic substances in the urine." The procedure is as follows: 10 cc. of urine are placed in a 50 cc. flask, silver lactate and colloidal iron are added, and the flask is filled to the mark. By this means uric acid and traces of protein which give a blue color with the reagent are removed. The contents are filtered, 25 cc. of the filtrate transferred to a 50 cc. flask, and sodium chloride is added to precipitate the excess of silver. The flask is filled to the mark and the contents are again filtered. The free phenols are determined by taking 20 cc. of this filtrate in a volumetric flask and adding sodium carbonate and the reagent. The blue color developed is compared with that given by a standard solution of phenol. The total phenols are determined by transferring a second 20 cc. of the filtrate to a large test-tube and adding 10 drops of concentrated HCl. The test-tube is covered with a small funnel and the mixture heated to boiling over a free flame and then placed in boiling water for 10 minutes. After cooling, the filtrate is treated as in the determination of the free phenols and the result obtained is a measure of the free plus conjugated phenolic substances. With normal adults the values given by Folin and Denis[3] for the free phenolic substances varies from 170 to 290 mg. per day and the total from 290 to 480 mg. per day.[4]

During the past year a great number of determinations had been made by the Folin and Denis method in this laboratory on the urine of infants and children and it was thought desirable to investigate the direct production of these substances by various bacteria. An obstacle was immediately encountered in the fact that tyrosine also reacts with the phenol reagent. In the effort to devise a method of separating the phenolic substances from tyrosine it was found that *p*-oxybenzoic acid, *p*-cresol, and phenol could be quantitatively recovered from an aqueous solution by shaking with two or more times its volume of ether and then extracting from the ether with 10 per cent NaOH. The NaOH was neutralized and the phenolic substances were estimated by the addition of sodium carbonate and the phenol reagent. This procedure was then tried out on urine and the results obtained (Table I) were so much lower than those by the Folin and Denis method that the reason for this was investigated.

---

[3] Folin and Denis (2), pp. 313, 316, 318.

[4] Considerable work has been done by Dubin (44) on the elimination of phenolic substances in the urine of animals using the Folin and Denis method.

It is primarily necessary to demonstrate that in the determinations made by the ether extraction method all the phenolic substances are quantitatively extracted and not destroyed during this and the subsequent treatment.

TABLE I.

Estimation of Phenolic Substances in Urine by Folin and Denis' Method and by Ether Extraction.

Case.	24 hr. quantity of urine.	Free phenolic substances.			Total phenolic substances.		
		Reaction at which extractions were made.	Folin and Denis' method.	Ether extraction method.	Reaction at which extractions were made.	Folin and Denis' method.	Ether extraction method.
	cc.	*pH*	*mg.*	*mg.*	*pH*	*mg.*	*mg.*
T.	775	5.6	210	60	1	388	151
J.	575	4.8	168	61	1	250	117
W.	830	5.6	227	82	1	340	167
H.	730	3.6	234	76	1	340	145

Tyrosine and uric acid are insoluble in ether, therefore the extractions may be made directly on the urine. The procedure is as follows: 5 cc. of urine are shaken for 5 minutes with 100 cc. of ether. The urine is separated and two more extractions are made using 50 cc. of ether each time. The 200 cc. of ether are shaken for 5 minutes with 20 cc. of 10 per cent NaOH, separated, and the sodium hydroxide solution is neutralized and made slightly acid with concentrated HCl. Sodium carbonate and the phenol reagent are then added and the color produced is compared with a standard phenol solution.

All the phenolic substances in the urine are many times more soluble in ether than in water and if present in the urine in their free state and not combined with other substances would be readily extracted by the relatively large amount of ether used.

That practically all the free phenolic substances are extracted is shown by making a fourth extraction on the 5 cc. of urine and after the subsequent shaking with NaOH only a barely perceptible color is developed on the addition of the reagent. Anderson (45) has called attention to the difficulty of completely removing *p*-cresol from ether by extraction with NaOH. That this difficulty does not exist when working with very small quantities is shown by making a second extraction on the 200 cc. of ether with 10 per cent NaOH and on adding the phenol reagent to it no color is produced.

It is seen that the removal of the phenolic substances from ether depends on converting them into their sodium salts which are insoluble in ether; therefore if these substances were present in the urine in the form of salts they would not be extracted. A survey of the properties of the phenolic substances soon shows that with the degree of acidity at which the extractions were made these substances would be present in their free state. At pH 6 all the volatile phenols would be free because sodium phenolate is largely hydrolyzed into sodium hydroxide and phenol even in a slightly alkaline solution. The question of the oxy-acids, however, is a different one as on account of their carboxyl group they behave as much stronger acids than phenol. That they are completely extracted at pH 6 is shown by the following experiment.

Two equivalents of NaOH were added to *p*-oxybenzoic acid (Kahlbaum). The resulting solution had a pH of 8^+. 5 cc. of this solution were brought to pH 7.4 and shaken for 5 minutes with 100 cc. of ether, and the ether was separated and shaken for 5 minutes with a 20 per cent solution of sodium carbonate. On the addition of the phenol reagent no color was obtained. This procedure was then repeated on a second 5 cc. of the sodium oxybenzoate solution which had been brought to pH 6.4, and on the addition of the reagent a marked blue color developed, which accounted for all the *p*-oxybenzoic acid in the solution.

There is also the possibility of actual free phenolic substances not being extracted by ether by virtue of some loose chemical combination in the urine or a possible adsorption with colloidal material. This is very improbable, however, and could not possibly account for the great difference obtained by the two methods.

The phenolic substances are not destroyed by the ether extraction and the subsequent steps of this method. This has already been shown in aqueous solutions with *p*-oxybenzoic acid, *p*-cresol, and phenol. If 5 cc. of urine after being used for the extraction with ether are treated by Folin and Denis' method, the amount of the so called phenolic substances obtained plus the amount obtained by ether extraction is only from 5 to 10 per cent less than that obtained on the urine directly. In view of the amount of manipulation these results correspond very closely and show that the phenolic substances are not destroyed.

Folin and Denis[3] explained the high results obtained by their method as due to the elimination of "a preliminary (necessarily imperfect) separation by [ether] extraction or distillation as has been the case in all the methods hitherto available." They conclude that the large amount of free phenol excreted after benzene has been given is "an interesting and probably convincing proof that the phenols formed within the body are in fact eliminated as free phenols." The methods of Kossler and Penny, Neuberg, Mooser, etc. require a preliminary evaporation of the faintly alkaline urine to one-fifth its original volume. Folin and Denis demonstrated that in an aqueous solution phenol and *p*-cresol are largely destroyed by evaporation for 1 hour after the addition of 1 drop of 10 per cent NaOH. Hence the presumed discrepancy between the higher results obtained by Folin and Denis and those of the older investigators.

Kossler and Penny's method was devised in 1893 and during the 42 years previous to this it was demonstrated time and again that only a trace of volatile phenols was present in the free state. It is of interest to note the results obtained by Munk in 1876 after giving benzene to an adult and estimating the volatile phenols by Landolt's method. The total urine for the day was distilled without acidifying on the sand bath. On the addition of bromine water to the distillate only a trace of volatile phenols was demonstrated. The total urine was again distilled after the addition of H_2SO_4 and on then adding bromine water to the distillate 101 mg. of tribromophenol were obtained. On repeating this experiment Folin and Denis found by their method that "there was an unmistakable increase in the elimination of the free as well as the conjugated phenols."

That the volatile phenols (and traces of non-volatile phenols) are only excreted in very small quantities in the free state in normal human urine is shown by the following experiments.

250 cc. of fresh urine were made alkaline (pH 7.4) by the addition of 10 per cent NaOH. The amount of alkali required in four different specimens were 5, 2, 2.7, and 3.6 cc. The urine was placed on the steam bath and evaporated to 50 cc. which required from 1 to 1½ hours. It was then made up to its original volume with water and the phenolic substances were estimated by Folin and Denis' method. The results obtained were invariably found to be the same as before evaporation. Folin and Denis demonstrated that free volatile phenols were largely destroyed by this treatment.

250 cc. of fresh urine were placed in a liter Kjeldahl flask and gently distilled for 4 hours. To a portion of the distillate were added sodium carbonate and the phenol reagent and the resulting color was read in the colorimeter. The free volatile phenols found in two different 24 hour specimens were 2.6 and 1.2 mg. To another 250 cc. of urine from one of the above specimens were added 2 mg. of phenol and the distillation was carried out under the same conditions. The amount of phenol recovered in the distillate in addition to the amount already found was 1.74 mg. or 87 per cent. The acidity of both urines was greater than pH 6.

Duplicate samples of urine were extracted with ether. To one extract were added 20 cc. of 20 per cent sodium carbonate and to the other 20 cc. of 10 per cent NaOH. The difference in the amount of color developed is a measure of the free volatile phenols present. The results obtained on five normal urines from adults (Table II) show that with the exception of one determination there was only a small amount of free volatile phenols present.

If ether contains aromatic oxy-acids, and volatile and non-volatile phenols, the addition of NaOH removes all these substances, but if sodium carbonate is added in place of the NaOH only the oxy-acids are removed. It is thereby possible to estimate the amount of volatile and non-volatile phenols in urine.

All the urine in the above determinations was preserved with a slight amount of chloroform and used immediately on completion of the 24 hour period. This is necessary as the free oxy-acids are rapidly converted into free *p*-cresol and phenol if the urine is allowed to stand. In this regard it is rather interesting to find, in confirmation of Baumann's work, that at the end of 5 or 6 days there are no free oxy-acids in the urine and their place is largely taken by the volatile phenols.

These three experiments prove conclusively that the volatile phenols are present in the urine largely in the conjugated form in contradiction to the results obtained by the Folin and Denis method.

It is of interest to estimate the total amount of volatile phenols in the urine by direct ether extraction and compare the results obtained with those recorded in the literature. The "deconjugation" was carried out exactly as outlined in Folin and Denis' method. The addition of the HCl brought the pH to about 1. The extraction may be carried out on the urine directly or on Folin and Denis' second filtrate. The latter procedure is the preferable one as the solution does not tend to adhere to the sides of the separatory funnel. The results obtained (Table II) are seen to have almost the identical range as those obtained by Mooser.

There are two sources of error in these determinations of the total volatile phenols. The first error is that some of the *p*-cresol is lost in the heating necessary for the liberation from the sulfuric and glucuronic acids. If *p*-cresol in amounts varying from 0.1 to 0.5 mg. is added to the solution being heated, it is found that about 25 per cent is not recovered. When phenol is added it is all recovered. As the amount of *p*-cresol in human urine is over 50 per cent of the volatile phenols this error could then be regarded as from 10 to 15 per cent, but, as the *p*-cresol is gradually being liberated during the heating, I think it is reasonable to regard this error as being in the neighborhood of 5 per cent. The second error is that any trace of pyrocatechin present in the urine would also be extracted. As these two errors tend to balance each other, the results obtained may be regarded as a comparatively accurate measure of the volatile phenols in the urine.

The amount of aromatic oxy-acids estimated as phenol is given in Table II. These results are in accord with the finding of Baumann in 1876 in which he demonstrated that the oxy-acids were largely excreted in the free state. It must be remembered, however, that the actual amount of oxy-acids is much greater than these figures as it requires about 5 mg. of *p*-oxybenzoic acid to produce the same amount of color as 1 mg. of phenol.

What the interfering substances are which react with the phenol reagent have not been determined. Some are liberated by the heating with concentrated HCl and this suggests that these may in part be conjugated. Indoxyl, which when conjugated with sulfuric acid produces indoxyl sulfate, has the benzene ring and a free hydroxyl group, but this group is not attached to the benzene nucleus. Besides Folin and Denis state that the phenol reagent does not react with indole or its derivatives. Muconic acid, which is an oxidation product of benzene, suggests itself, as Munk (13) found on giving benzene no increase in the free volatile phenols while Folin and Denis found a marked increase. Muconic acid, however, is soluble in ether and would therefore be extracted. Inosite, $C_6H_6(OH)_6$, which is a polyhydric alcohol, is insoluble in ether and present in large quantities in urine but does not react with the phenol reagent.

TABLE II.

Estimation of Free and Total Volatile Phenols and Aromatic Oxy-Acids by Ether Extraction.

Case.	24 hr. quantity of urine.	Free volatile phenols.	Total volatile phenols.	Free aromatic oxy-acids estimated as phenol.	Total aromatic acids estimated as phenol.
	cc.	*mg.*	*mg.*	*mg.*	*mg.*
F. F. T.	760	9		60	75
F. F. T.	775	2	58	60	93
J.	575	6	40	55	77
W.	830	17	40	65	127
H.	730	0	38	76	107

Some of the properties of the interfering substances have been determined. If fresh urine is distilled for some hours with phosphoric or sulfuric acid until all the volatile phenols have been driven off, the distillate contains a compound which reacts not only with the phenol reagent but also with Folin and Macallum's (46) uric acid reagent and Folin and Wu's (47) new blood sugar reagent. These substances distil over in large quantities, particularly if the fluid in the distilling flask is allowed to become concentrated. Also, if uric acid and traces of protein are removed from urine and the filtrate is heated with concentrated HCl, the addition of the uric acid and sugar reagents produces a considerable color. The reaction with the sugar reagent is very slow, the color increasing in intensity for 2 or 3 days. Folin and

Wu state that the blood sugar reagent does not react with "phenols." The color produced by the uric acid reagent might be accounted for by pyrocatechin, as this reagent reacts with the polyphenols.

SUMMARY.

1. The results obtained for the total phenolic substances in urine by an ether extraction method outlined in this paper are at least 50 per cent lower than those recorded by Folin and Denis.
2. Only a small fraction of the volatile phenols are excreted in the urine in the free state.
3. The amount of volatile phenols found by the method described in this paper corresponds very closely with the values obtained by the methods of Mooser and Hensel.
4. There are substances in the urine, at present unidentified, which react with the phenol, uric acid, and blood sugar reagents devised by Folin and his collaborators. These compounds are partly present in the free state and partly formed by the action of strong mineral acids on the urine. Whether they are simple conjugated bodies or chemical compounds formed by the action of strong mineral acids on some constituent of the urine has not been determined.
5. It is suggested that these compounds may be responsible for the high values obtained for the phenolic substances in urine by the method of Folin and Denis.

Addendum.—After the completion of this manuscript there appeared a paper on the colorimetric estimation of tyrosine by the method of Folin and Denis (48). In investigating Folin and Denis' phenol reagent, the authors found that tryptophane also produced an intense blue color with the reagent. Indole and indole derivatives, contrary to the statement of Folin and Denis, reacted strongly with the reagent to produce the blue color. They found considerable evidence that tyrosine and tryptophane were not the only protein constituents which produced blue colors with the reagent and also that ferrous iron and apparently any other easily oxidizable material caused the reaction.

BIBLIOGRAPHY.

1. Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.
2. Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 311.
3. Städeler, G., *Ann. Chem.*, 1851, lxxvii, 17.
4. Schultzen, O., and Naunyn, B., *Arch. Anat. u. Physiol.*, 1867, 349.
5. Landolt, H., *Ber. chem. Ges.*, 1871, iv, 770.

6. Brieger, L., *Z. physiol. Chem.*, 1880, iv, 207.
7. Baumann, E., *Z. physiol. Chem.*, 1879, iii, 251, 252.
8. Buliginsky, A., *Med. chem. Untersuch.*, 1867, 234.
9. Hoppe-Seyler, F., *Arch. ges. Physiol.*, 1872, v, 470.
10. Baumann, E., *Arch. ges. Physiol.*, 1876, xii, 69.
11. Baumann, E., *Arch. ges. Physiol.*, 1876, xiii, 293, 295.
12. Schmiedeberg, O., *Arch. exp. Path. u. Pharmakol.*, 1881, xiv, 307.
13. Munk, I., *Arch. ges. Physiol.*, 1876, xii, 142.
14. Baumann, E., *Z. physiol. Chem.*, 1877, i, 61, 65.
15. Baumann, E., and Brieger, L., *Z. physiol. Chem.*, 1879, iii, 149.
16. Weyl, T., *Z. physiol. Chem.*, 1879, iii, 312.
17. Brieger, L., *Ber. chem. Ges.*, 1877, x, 1027.
18. Brieger, L., *Z. physiol. Chem.*, 1878, ii, 250.
19. Hoppe-Seyler, G., *Z. physiol. Chem.*, 1888, xii, 1.
20. Baumann, E., *Z. physiol. Chem.*, 1886, x, 127, 129.
21. Ewald, C. A., *Virchows Arch. path. Anat.*, 1879, lxxv, 416.
22. Ebstein, W., and Müller, J., *Virchows Arch. path. Anat.*, 1875, lxii, 554.
23. Baumann, E., *Arch. ges. Physiol.*, 1876, xii, 63, 67.
24. Baumann, E., and Preusse, C., *Z. physiol. Chem.*, 1879, iii, 157.
25. Nencki, M., and Giacosa, P., *Z. physiol. Chem.*, 1880, iv, 336.
26. Baumann, E., *Ber. chem. Ges.*, 1879, xii, 1452.
27. Baumann, E., *Ber. chem. Ges.*, 1880, xiii, 279, 283.
28. Baumann, E., *Z. physiol. Chem.*, 1880, iv, 307, 309, 310, 312.
29. Salkowski, H., *Ber. chem. Ges.*, 1879, xii, 1438.
30. Weyl, T., *Ber. chem. Ges.*, 1879, xii, 354.
31. Schultzen, O., and Riess, L., Jahresberichte der Chemie, 1869, 810.
32. Baumann, E., *Z. physiol. Chem.*, 1882, vi, 193.
33. Munk, I., *Arch. Physiol.*, 1880, suppl., 28.
34. Koppeschaar, W. F., *Z. anal. Chem.*, 1876, xv, 233.
35. Kossler, A., and Penny, E., *Z. physiol. Chem.*, 1893, xvii, 117.
36. Messinger, J., and Vortmann, G., *Ber. chem. Ges.*, 1889, xxii, 2313.
37. Neuberg, C., *Z. physiol. Chem.*, 1899, xxvii, 123.
38. Mooser, W., *Z. physiol. Chem.*, 1909, lxiii, 155.
39. Neuberg, C., and Hildesheimer, A., *Biochem. Z.*, 1910, xxviii, 525.
40. Hensel, M., *Z. physiol. Chem.*, 1912, lxxviii, 373.
41. Bordas, F., and Robin, L., *Compt. rend. Soc. biol.*, 1898, v, Series 10, 87.
42. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.
43. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 106.
44. Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69; 1917, xxxi, 255.
45. Anderson, R. J., *J. Biol. Chem.*, 1916, xxvi, 387.
46. Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912–13, xiii, 363.
47. Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.
48. Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1920, lxii, 1678.

COMPOSITION OF INOSITE PHOSPHORIC ACID OF PLANTS.

SEVENTEENTH PAPER ON PHYTIN.

BY R. J. ANDERSON.

(*From the Biochemical Laboratory, New York Agricultural Experiment Station, Geneva.*)

(Received for publication, August 6, 1920.)

INTRODUCTION.

A number of investigators have isolated and studied inosite phosphoric acid compounds derived from seeds and various feeding material.[1] Unfortunately there is no agreement among these investigators as to the composition of the phytic acid or inosite phosphoric acid, nearly every worker in this field having found one or more substances which differ in composition from those analyzed by others.

Posternak[2] who first carefully studied this substance concluded from his data of the analyses of crystalline double calcium-sodium salts that phytin was a salt of an acid having the formula $C_2H_8O_9P_2$. On theoretical grounds Neuberg[3] suggested that this formula should be multiplied by 3; *viz.*, $C_6H_{24}O_{27}P_6$. On equally theoretical grounds it was assumed by Suzuki and collaborators[4] that the acid was a hexaphosphoric acid ester of inosite, $C_6H_{18}O_{24}P_6$. Starkenstein,[5] Vorbrodt,[6] and others elaborated more complex formulas for the acid. Patten and Hart[7] and Hart and Tottingham[8] regarded the substances which they analyzed as identical with the phytin of Posternak.

---

[1] For review of the literature see Rose, A. R., *Biochem. Bull.*, 1912–13, ii, 21. Anderson, R. J., Thesis, Cornell University, 1919.

[2] Posternak, S., *Compt. rend. Acad.*, 1903, cxxxvii, 202, 337, 439.

[3] Neuberg, C., *Biochem. Z.*, 1908, ix, 557.

[4] Suzuki, U., Yoshimura, K., and Takaishi, M., *Bull. Tokyo Imper. Univ. College Agric.*, 1907, vii, 503.

[5] Starkenstein, E., *Biochem. Z.*, 1911, xxx, 56.

[6] Vorbrodt, W., *Bull. de l'Acad. sc. Cracow, Series A*, 1910, 414.

[7] Patten, A. J., and Hart, E. B., *Am. Chem. J.*, 1904, xxxi, 564.

In earlier papers from this laboratory[9] it was assumed that the phytic acid formula according to Posternak and Neuberg was correct and the analytical data from amorphous phytin preparations were in close agreement with this formula. But in isolating the organic phosphoric acid of cotton-seed meal[10] we were able to obtain crystalline barium salts which corresponded closely in composition to salts of inosite hexaphosphoric acid. Identical crystalline salts were later obtained from oats, corn, commercial phytin,[11] wheat bran,[12] and more recently from maple seed.[13]

These barium salts had been very carefully purified by repeated recrystallization until the composition remained constant. Two types of barium salts were obtained corresponding to the following formulas, $C_6H_{12}O_{24}P_6Ba_3$ and $(C_6H_{11}O_{24}P_6)_2Ba_7$. The first or tribarium inosite hexaphosphate crystallizes slowly in rosettes of microscopic needles after addition of alcohol to a solution of the barium salt in dilute hydrochloric acid. The second salt, which may be regarded either as a heptabarium inosite hexaphosphate or as a mixture of equal parts of tri- and tetrabarium inosite hexaphosphate, is obtained on heating a solution of the barium salt in dilute hydrochloric acid in the presence of an excess of barium chloride or it separates slowly as a heavy powder or crystalline crust when a dilute hydrochloric acid solution of the barium salt containing a large excess of barium chloride is allowed to stand at room temperature.

Thompson[14] obtained similar crystalline barium salts from the inosite phosphoric acid of rice bran and Robinson and Mueller[15] from wheat bran. Clarke[16] and Boutwell[17] using similar methods isolated substances from wild Indian mustard and wheat bran which differed considerably in composition from salts of inosite hexaphosphoric acid. Rather[18] has expressed the opinion that the preparations which he had isolated were not salts of inosite hexaphosphoric acid but of acids represented by various formulas such as $C_4H_{17}O_{15}P_3$, $C_6H_{17}P_4O_{17}$, $C_4H_{15}P_3O_{13}$, but later he believed that the composition was best represented by the formula $C_{12}H_{41}O_{42}P_9$. In a more recent publication[19] it is stated by him that the composition of the inosite phosphoric acid of plants is equally well expressed either by the formula $C_{12}H_{41}O_{42}P_9$ or by the formula of inosite pentaphosphoric acid, $C_6H_{17}O_{21}P_5$.

---

[8] Hart, E. B., and Tottingham, W. E., *J. Biol. Chem.*, 1909, vi, 431.

[9] Anderson, R. J., *J. Biol. Chem.*, 1912, xi, 471; xii, 97; *New York Agric. Exp. Station, Tech. Bull. 19, 21*, 1912.

[10] Anderson, R. J., *J. Biol. Chem.*, 1912-13, xiii, 311; 1914, xvii, 141; *New York Agric. Exp. Station, Tech. Bull. 32*, 1914.

[11] Anderson, R. J., *J. Biol. Chem.*, 1914, xvii, 151, 165, 171; *New York Agric. Exp. Station, Tech. Bull. 32*, 1914.

[12] Anderson, R. J., *J. Biol. Chem.*, 1915, xx, 493; *New York Agric. Exp. Station, Tech. Bull. 40*, 1915.

[13] Anderson, R. J., *J. Biol. Chem.*, 1920, xliii, 469; *New York Agric. Exp. Station, Tech. Bull. 81*, 1920.

[14] Thompson, A. R., *J. Agric. Research*, 1914-15, iii, 425.

[15] Robinson, C. J., and Mueller, J. H., *Biochem. Bull.*, 1915, iv, 100.

It is possible that substances having the composition described by Rather may be isolated from plant material. We believe, however, when inosite penta-, tetra-, or other lower inosite phosphoric acids are found that they have been formed through partial hydrolysis of the inosite hexaphosphoric acid. Such partial hydrolysis may occur either in the plant material itself during storage as found by Rather[20] in cotton-seed meal and by the writer[13] in maple seed or else it may occur during the process of isolating the substance through enzymotic action when the material is digested in water as shown by Suzuki, Yoshimura, and Takaishi,[4] by the writer,[21] and by Boutwell[17] on digestion in very dilute acids.

Moreover, the analytical data presented by Rather do not prove conclusively that the composition of the inosite phosphoric acid of plants corresponds to inosite pentaphosphoric acid. It was suggested by the writer some years ago that the strychnine salts[22] are not suitable for the identification of inosite phosphoric acid because the great basicity of the acid gives salts of very high molecular weight. Slight differences in composition of the acid, therefore, cannot be determined by analysis of such salts for the reason that analytical errors may be greater than the difference in calculated percentage composition. This is illustrated in Table I. In the second column is given the average of the sixteen strychnine salts analyzed by Rather; in the third column the calculated composition of tetrastrychnine inosite pentaphosphate; and in the fourth column the calculated percentage of a mixture assumed to consist of two parts of pentastrychnine inosite hexaphosphate and one part of tetrastrychnine inosite hexaphosphate.

---

[16] Clarke, G., *J. Chem. Soc.*, 1914, cv, 535; 1915, cvii, 360.

[17] Boutwell, P. W., *J. Am. Chem. Soc.*, 1917, xxxix, 491.

[18] Rather, J. B., *Texas Agric. Exp. Station, Bull. 146*, 1912; *J. Am. Chem. Soc.*, 1913, xxxv, 890.

[19] Rather, J. B., *J. Am. Chem. Soc.*, 1918, xl, 523.

[20] Rather, J. B., *J. Am. Chem. Soc.*, 1917, xxxix, 777.

[21] Anderson, R. J., *J. Biol. Chem.*, 1915, xx, 483; *New York Agric. Exp. Station, Tech. Bull. 40*, 1915.

[22] Anderson, R. J., *J. Biol. Chem.*, 1915, xx, 463; *New York Agric. Exp. Station, Tech. Bull. 40*, 1915.

It is self-evident that such slight differences in percentage composition as exist between the above formulas cannot be determined accurately by analysis. Having due regard to the limit of error in analytical work we do not believe that the data of the above author present any definite proof that inosite pentaphosphoric acid exists in plants.

TABLE I.

Comparative Composition of Strychnine Salts.

	Average of sixteen analyses reported by Rather.	Calculated for tetrastrychnine inosite pentaphosphate $C_6H_{17}O_{21}P_5(C_{21}H_{22}N_2O_2)_4$.	Calculated for $2[C_6H_{18}O_{24}P_6(C_{21}H_{22}N_2O_2)_5]$ $+C_6H_{18}O_{24}P_6(C_{21}H_{22}N_2O_2)_4$.
	per cent	*per cent*	*per cent*
C	55.99	56.36	56.25
H	5.76	5.48	5.43
N	5.46	5.84	5.88
P	8.45	8.08	8.38

Posternak[23] has recently called attention to his earlier work and again he describes the crystalline double calcium-sodium salt which he analyzed several years ago. At the present time this author accepts the phytic acid formula proposed by Neuberg; *viz.*, $C_6H_{24}O_{27}P_6$. This formula differs from that of inosite hexaphosphoric acid by containing 3 molecules more of H_2O, $C_6H_{18}O_{24}P_6 + 3H_2O = C_6H_{24}O_{27}P_6$. In what manner these 3 molecules of water are combined in the acid is not explained. The above author not only believes that the phytic acid existing in plant material has this formula but he claims to have synthesized an identical acid[24] by heating a mixture of inosite, phosphoric acid, and phosporus pentoxide.

---

[23] Posternak, S., *Compt. rend. Acad.*, 1919, clxviii, 1216; clxix, 37.

On repeating these experiments on the synthesis of phytic acid, the writer[25] was unable to confirm the conclusion of Posternak that inosite hexaphosphoric acid or phytic acid was formed. The only product which he could isolate from the reaction mixture corresponded to the formula $C_6H_{12}O_{18}P_4$, which is evidently an inosite ester of pyrophosphoric acid. This new acid contains very nearly the same percentage of phosphorus as phytic acid but in properties and reactions it differs from this substance in several particulars.

In view of the disparity in the results of the several investigators, Patten and Hart, Clarke, Boutwell, Rather, Robinson and Mueller, and the writer, as to the composition of the phytic acid of wheat bran this substance has been reinvestigated.

It was noticed in this laboratory when wheat bran is digested in about 0.2 per cent hydrochloric acid that compounds differing in composition from inosite hexaphosphoric acid are obtained.[26] From this mixture of organic phosphoric acids we were able to identify two, inosite monophosphoric acid[27] and inosite triphosphoric acid.[22] It was found later, however, on digesting wheat bran in 0.5 or 1.0 per cent hydrochloric acid that salts of inosite hexaphosphoric acid[12] were obtained and it was also found that the stronger acid destroyed the enzyme, phytase, which hydrolyzed the inosite hexaphosphoric acid when the bran was digested in 0.2 per cent hydrochloric acid.

In the present investigation it was found that the crystalline barium salts corresponded very closely in composition to those previously obtained from this material. The work fully confirms not only our earlier results with respect to the composition of the phytic acid of wheat bran, but the results are also in agreement with the preparations isolated from cotton-seed meal, commercial phytin, oats, corn, and maple seed which have been reported from this laboratory.

---

[24] Posternak, S., *Compt. rend. Acad.*, 1919, clxix, 138.

[25] Anderson, R. J., *J. Biol. Chem.*, 1920, xliii, 117; *New York Agric. Exp. Station, Tech. Bull. 79*, 1920.

[26] Anderson, R. J., *J. Biol. Chem.*, 1912, xii, 447; 1914, xviii, 425; *New York Agric. Exp. Station, Tech. Bull. 22*, 1912; *Bull. 36*, 1914.

[27] Anderson, R. J., *J. Biol. Chem.*, 1914, xviii, 441; *New York Agric. Exp. Station, Tech. Bull. 36*, 1914.

The relation of carbon to phosphorus in all these carefully purified and many times recrystallized barium salts is in the ratio of 6 : 6. The percentage of phosphorus is slightly higher and the percentage of carbon is slightly lower than is required by the phytic acid formula of Posternak. We have been forced to the conclusion, therefore, that the assumption of Suzuki, Yoshimura, and Takaishi that phytic acid is inosite hexaphosphoric acid, $C_6H_{18}O_{24}P_6$, is correct.

EXPERIMENTAL.

Preparation of the Barium Salt.

The usual procedure of isolating the substance from the wheat bran was slightly modified. The following method, which was found to be the most satisfactory, is here briefly described. The wheat bran, 3 kilos, was digested in 12 liters of 2 per cent hydrochloric acid for 5 hours with frequent stirring. It was then strained through cheese-cloth and the extract was freed from suspended starch, etc., by centrifuging and finally by filtering through a layer of paper pulp. To the clear filtrate was added sufficient sodium acetate to bind the free hydrochloric acid. The phosphorus compounds were then precipitated by adding a concentrated solution of barium chloride until no further precipitate was produced. After settling, the supernatant liquid was syphoned off and the barium salt was freed from the mother liquor by centrifuging and then filtering and washing with water on a Buchner funnel.

The washed precipitate was suspended in water and the barium was removed by adding a slight excess of sulfuric acid. After the barium sulfate had settled, it was filtered off and the clear filtrate was precipitated by adding a solution of copper acetate. The resulting copper salt was filtered and washed free from sulfates with water; then it was suspended in water and decomposed with hydrogen sulfide. The copper sulfide was removed and the filtrate was freed from excess of hydrogen sulfide with a current of air and then precipitated by adding a solution of barium hydroxide. The barium salt was filtered and washed thoroughly with water. It was dissolved in dilute hydrochloric acid, filtered, and reprecipitated with barium hydroxide. After precipitating in this manner seven times with barium hydroxide, the substance was precipitated four times with alcohol from dilute hydrochloric acid. It was finally filtered and washed free from chlorides with dilute alcohol, and alcohol and ether, and dried in vacuum over sulfuric acid. The substance was a snow-white, bulky, crystalline powder and it gave no reaction with ammonium molybdate, indicating absence of inorganic phosphate. It weighed 95 gm. Bases other than barium were absent.

For the purpose of removing any oxalic acid which might have been retained, the substance was dissolved in dilute hydrochloric acid and to it was added a concentrated solution of 20 gm. of barium chloride. After standing over night the slight precipitate which had formed was filtered off and the filtrate precipitated by adding an equal volume of 95 per cent alcohol. The substance was filtered and washed in dilute alcohol until free from chlorides and then washed in alcohol and ether and dried in vacuum over sulfuric acid.

The substance was further crystallized as follows: It was dissolved in dilute hydrochloric acid and the free acid nearly neutralized by adding with constant shaking a dilute solution of barium hydroxide until a faint permanent cloudiness remained. The solution was filtered and alcohol was added slowly with shaking until a slight permanent precipitate was produced. The solution was again filtered, a few grams of barium chloride were added, and the whole was allowed to stand at room temperature for 24 hours. The barium salt separated slowly as a compact mass consisting of rosettes or globular masses of microscopic needles. The substance was recrystallized in this manner three times. The crystalline precipitate was finally washed in dilute alcohol until free from chlorides and then in absolute alcohol and ether. For analysis portions of this material were further recrystallized as follows.

Preparation of Heptabarium Inosite Hexaphosphate.

10 gm. of the above substance were dissolved in dilute hydrochloric acid, the free acid was nearly neutralized with a dilute solution of barium hydroxide, and the solution filtered. To this

were added 10 gm. of barium chloride and the solution was allowed to stand at room temperature for 2 days. The substance separated slowly as a heavy compact crystalline crust which consisted of small rosettes of microscopic needle-shaped crystals. It was recrystallized a second time in the same way and was then filtered, washed with water until free from chlorides, and finally washed in alcohol and ether and dried in the air.

For analysis the substance was dried at 105° in vacuum over phosphorus pentoxide. It lost on drying 10.91 per cent of H_2O.

Found: $C = 6.25$; $H = 1.16$; $P = 16.32$; $Ba = 42.55$ per cent.

A second preparation recrystallized as above gave the following result.

Found: $C = 6.42$; $H = 1.22$; $P = 16.34$; $Ba = 42.55$; $H_2O = 9.71$ per cent.

Calculated for $(C_6H_{11}O_{24}P_6)_2\ Ba_7 + 14\ H_2O$: $C = 6.35$; $H = 0.97$; $P = 16.40$; $Ba = 42.39$; $H_2O = 10.00$ per cent.

Preparation of Tribarium Inosite Hexaphosphate.

10 gm. of the purified barium salt were dissolved in dilute hydrochloric acid and the filtered solution was precipitated by adding an equal volume of 95 per cent alcohol. The amorphous precipitate after standing for about 24 hours in contact with the mother liquor assumes a crystalline form which appears to be identical with that described above; *i.e.*, it consists of very minute rosettes of microscopic needles. It was then filtered and washed with 30, 50, and 95 per cent alcohol until free from chlorides and after displacing the alcohol with ether the substance was dried in vacuum over sulfuric acid. The product was recrystallized three times in this manner. It was obtained as a snow-white, bulky, crystalline powder which gave no reactions for either chlorides or inorganic phosphate.

For analysis it was dried in vacuum at 105° over phosphorus pentoxide.

Found: $C = 6.73$; $H = 1.69$; $P = 16.93$; $Ba = 38.60$ per cent.
" $C = 6.79$; $H = 1.30$; $P = 17.10$; $Ba = 39.00$ " "

Calculated for $C_6H_{12}O_{24}P_6Ba_3$: $C = 6.75$; $H = 1.12$; $P = 17.44$; $Ba = 38.65$ per cent.

Preparation of the Silver Salt.

3 gm. of the recrystallized heptabarium salt were suspended in water and the barium was precipitated with a slight excess of sulfuric acid. After the barium sulfate had been filtered off, the solution was precipitated with copper acetate. The copper salt was filtered, washed, suspended in water, and decomposed with hydrogen sulfide. After the copper sulfide was filtered off and the excess of hydrogen sulfide was removed with a current of air a colorless solution of the free acid was obtained. 10 cc. of this filtrate were titrated with 0.1 N NaOH and the calculated quantity of ammonia was added to the balance of the solution. This produced a slightly alkaline reaction. Silver nitrate was added in slight excess. The heavy white precipitate of the silver salt was filtered and washed thoroughly in water and then dried in vacuum over sulfuric acid. The silver salt was a nearly white, heavy, amorphous powder.

It was analyzed after drying at 105° in vacuum over phosphorus pentoxide. It turned quite dark in color on drying.

Found: C = 3.82; H = 0.49; P = 9.56; Ag = 66.08 per cent.
P = 9.57; Ag = 66.18 " "
Calculated for $C_6H_6O_{24}P_6Ag_{12}$: C = 3.70; H = 0.30; P = 9.57; Ag = 66.64 per cent.

Another silver salt was prepared in the same way as above except that it was made from a phytic acid preparation which had been purified as a crystalline strychnine salt.

Found: C = 3.90; H = 0.54; P = 9.48; Ag = 66.56 per cent.

These preparations are apparently neutral silver salts of inosite hexaphosphoric acid. All other silver salts which have been prepared and analyzed have been acid salts. It was hoped that a neutral ester of inosite hexaphosphoric acid might be obtained by the action of methyl iodide upon the neutral silver salt. However, all attempts to prepare such an ester were unsuccessful and only strongly acid syrups were obtained.

SUMMARY.

Data obtained from the analyses of carefully purified and recrystallized barium salts of the organic phosphorus compound of wheat bran are in close agreement with the calculated composition of barium salts of inosite hexaphosphoric acid.

Silver salts were prepared from the acid and these were found to agree with the formula $C_6H_6O_{24}P_6Ag_{12}$.

The composition of phytic acid of plants as determined from the analyses of salts of this acid corresponds to inosite hexaphosphoric acid, $C_6H_{18}O_{24}P_6$ or $C_6H_6O_6(PO(OH)_2)_6$.

EXPERIMENTAL STUDIES ON GROWTH.

XVI. THE INFLUENCE OF BRAIN TISSUE, FREED FROM CHOLESTEROL, UPON THE GROWTH OF THE WHITE MOUSE.

By T. BRAILSFORD ROBERTSON and L. A. RAY.

(From the Department of Physiology and Biochemistry, University of Adelaide, South Australia, and the Department of Biochemistry, University of Toronto, Toronto.)

(Received for publication, August 8, 1920.)

Statement of the Problem.

There are many observations, hitherto isolated, which, when taken together, point towards the nervous system as the source of some very decisive stimulant of the growth of parenchymatous tissues.

Thus Child has shown that in planarians the metabolic gradient ascends as we approach the nervous centers (1). Loeb has shown that pieces of marine Planaria which contain nervous tissue regenerate more rapidly than those which do not (2). Morgan has shown that excision of an adjacent piece of the nerve cord in earthworms delays or prevents regeneration (3). The regeneration of the eye in crustaceans is similarly dependent upon the integrity of the optic ganglion (4).

On the other hand Friedenthal has drawn attention to a remarkable relationship which subsists between the ratio of brain weight to weight of living tissue (cephalization factor) and the longevity of any species of a given natural order (5). The weight of living tissues, exclusive of supporting tissues, is estimated to be proportional to the two-thirds exponent of the body weight (6). This relationship is illustrated by the data in Table I cited after Friedenthal.[1]

---

[1] The life duration of the mouse, computed from our own observations, has been added to the table. The estimate of human life duration is somewhat reduced.

Now we have shown (7, 8) that substances and conditions which promote longevity in mice also exert an influence upon their growth, an influence which is of such a character as to denote stimulation of the growth of parenchymatous tissues to the detriment of the growth of competing (sclerous) tissues. If, therefore, the possession of a high cephalization factor conduces to the longevity of animals we should also expect it to affect the contour of the growth curve, and the growth curve of animals to which substances have been administered which accelerate the growth of parenchyma should display departures from the normal curve approximating towards the growth curve of animals which possess a higher cephalization factor.

TABLE I.

Species.	Cephalization factor.	Maximal life duration (Hansemann).
	Mammals.	
		yrs.
Man	2.67 to 2.81	80 to 130
Elephant	1.24 " 1.34	90 " 100
Horse	0.43 " 0.57	45
Dog	0.34 " 0.51	12 to 20
Cat	0.29 " 0.34	20
Ox	0.3 " 0.4	30
Squirrel	0.16 " 0.2	6
Mouse	0.04	3
	Birds.	
Carrion crow	0.168	100 (?)
Parrots	0.147 to 0.177	100 (?)
Alpine crow	0.114	50
Finch	0.086	8
Pheasant	0.0343	15
Fowl	0.0249	10 to 20

Our quantitative data concerning the growth of animals are, unfortunately, so scanty that we possess but few standards of comparison. We are in a position, however, to compare the growth of an animal having a very low cephalization factor, namely the mouse, with that of human beings, possessing a very high cephalization factor. Such a comparison is graphically displayed in Figs. 1 and 2.

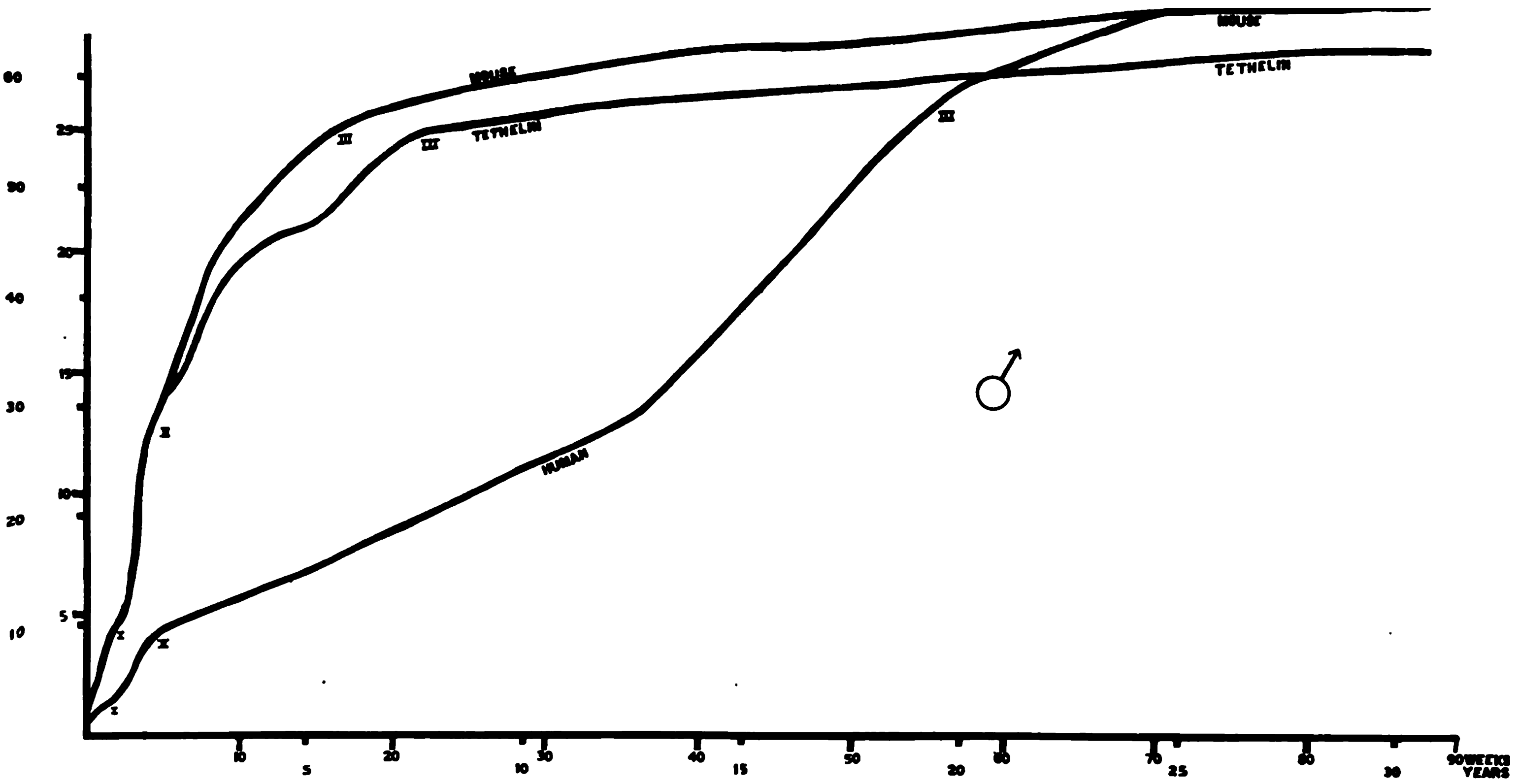


FIG. 1. A comparison of the curves of growth of male white mice and human beings. The administration of tethelin causes an approximation of the mouse curve towards the human curve.

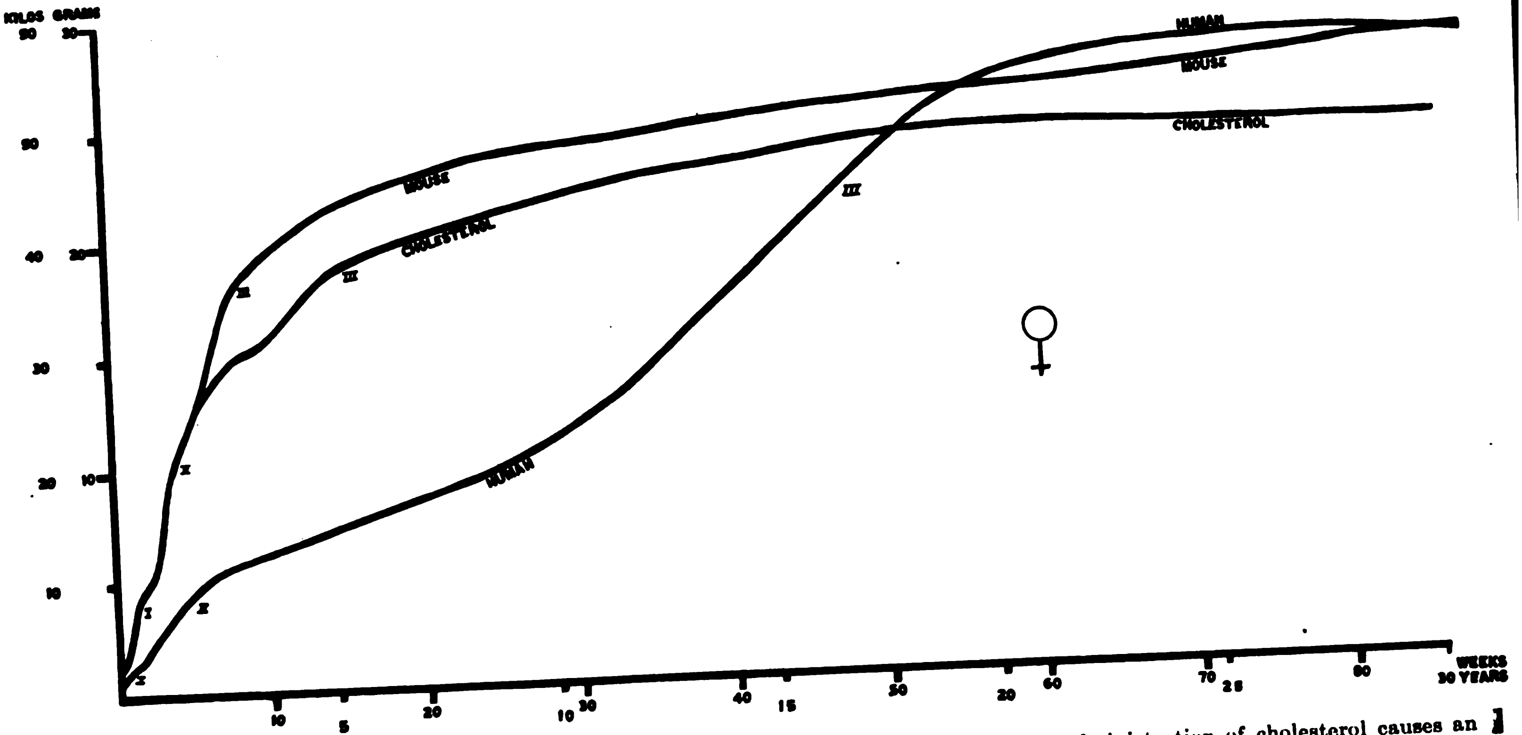


FIG. 2. A comparison of the curves of growth of female white mice and human beings. The administration of cholesterol causes an approximation of the mouse curve towards the human curve.

The data for the growth of human beings are derived from the measurements of Quetelet (9). More extensive measurements of human growth for restricted growth periods are available, but the measurements of Quetelet cover the whole period of growth, and in every feature which is at all essential to our comparison the curve of human growth, as depicted by Quetelet, has been confirmed by subsequent observations. The data for the growth of mice are taken from those for our "1914 normals," which have been published in a previous article of this series (10). The scales of weight and time are so chosen that the two curves reach the same level at approximately their maximum ordinates.

Each of the complete growth curves shows three waves or cycles of alternately rapid and slow growth (11). The position of these cycles is indicated in the figures by Roman numerals.

These comparisons at once reveal the fact that the human growth curve differs from the growth curve of the mouse in the relatively enormous prolongation of the second growth cycle and the great delay in the development of the third cycle. This period of delay is succeeded by a period of very rapid growth which culminates quickly in the maximum weight of maturity. The curve then declines very slowly towards senescence. The growth curve for the mouse, on the contrary, shows an abbreviated second cycle merging with hardly any pause or slackening of growth into the third cycle, which, however, does not quickly attain a maximum, but continues to display accretion of weight, until a relatively advanced age is attained. Thus a human being at 30 years of age has probably only lived for half his normal life duration (epidemics and accidents apart), while the mouse at 84 weeks of age has lived over four-fifths of its life. Nevertheless the human being has long since ceased to gain weight and the mouse is still growing, a phenomenon which is of significance in view of the fact that late accretion of weight in mice has been shown to be unfavorable to their longevity (8).

A comparison with the curve of growth for mice which have received 4 mg. of tethelin per day, which prolongs the duration of their life from 80 to 100 days, shows that the departures from the normal growth curves which are displayed in the growth of tethelin-fed mice are such as to bring the mouse curve into somewhat closer approximation to the human (Fig. 1). The same prolongation of the second cycle is seen, in lesser degree, and delay in the onset of the third cycle, succeeded by a measure of compensatory acceleration. Furthermore the later part of the curve remains parallel to the base line or descends, as in the human curve, indicating a diminished late accretion of tissue.

The growth curve of mice which have received 40 mg. of cholesterol per day (Fig. 2) resembles in general outline the growth curve of mice which have received 4 mg. of tethelin per day. The prolongation of life which might otherwise be displayed by these animals is, however, prevented by the secondary deleterious effects of the extensive deposits of cholesterol which are formed in various organs of animals receiving excessive amounts of cholesterol by mouth (12).

The facts which are brought out by this comparison are therefore in harmony with the view that the nervous system exerts an influence upon the growth of the other tissues of the body which is analogous in kind to that exerted by administrations of tethelin or cholesterol, but more intense in degree.

Two possible alternatives now present themselves; namely, (*a*) that the nervous system promotes the anabolism of parenchyma by the conduction of stimuli to it—the so called "trophic influence" of nerves, and (*b*) that the nervous system promotes the anabolism of parenchyma by the elaboration of a catalyzer which is liberated from the nervous tissues and officiates as a growth hormone.

It is exceedingly difficult with the information at present in our possession to distinguish between these two possibilities. The difficulty becomes apparent when we consider that the development of the catalyzer by the nervous tissues may be a by-product of, and therefore dependent upon, their activity as conducting tissues. It would be surprising if, in tissues so highly specialized for the purposes of conduction, an interruption of this function did not disturb or eventually transform their metabolic activities, and, indeed, the fact that the metabolic activities of nervous tissues are dependent upon their conducting function is shown by the phenomenon of secondary Wallerian degeneration.

On the other hand, the phenomena of dystrophy, following upon nerve section, would appear to militate against the chemical view of the trophic function of the nervous system, since a hormone liberated from nervous centers elsewhere might be expected, from the analogies provided by other hormones, to reach the tissues through the medium of the circulation and independently of their immediate nervous connections. The question is not settled by this simple consideration, however, for two possibilities still exist; namely, (1) that the basic generalized supply of hormone liberated by the nervous system is insufficient for the total needs of the tissues and must be supplemented by a local supply, and (2) that the hormone does not travel in the aqueous fluids of the body, but in the nerve trunks themselves (13), in this respect behaving analogously to tetanus toxin, which travels, however, centripetally from a peripheral source of supply (14), instead of centrifugally from a central source of supply as this hypothetical hormone would do. Needless to say, these possibilities are not mutually exclusive and both of them may be true, and it may also be true that a succession of nervous impulses is required to maintain the normal nutrition of tissues which, by specialization, have become dependent upon conducting tissues.

It is impossible to disentangle these various alternatives by any method other than that of direct experiment in which the several factors which we have enumerated are dissociated. While we cannot at present dissociate the stream of impulses impinging on a tissue from the metabolic activities of the nerve tissues which conduct them, we can administer to animals or to individual tissues the chemical components of nervous tissue in excess without modification of their nerve supply. The absorption of such substances from the alimentary tract presents no greater difficulty than the absorption of strychnine, which, shortly after administration by mouth, is found to be confined to the nervous tissues of the animal to which it has been administered, or than the absorption of cholesterol, so large a component of nervous tissues which is insoluble in water, but is nervertheless absorbed preformed (15) and carried to the nervous tissues in a condition of emulsification.

The effect of cholesterol itself upon the growth of animals is known (16, 17). As we have seen, the effect of 40 mg. of cholesterol daily is comparable to the effect of 4 mg. of tethelin and the effect of this, in turn, is far inferior to the influence of a high cephalization factor upon the time relations of growth in animals. Obviously, therefore, if a catalyzer of growth is manufactured by nervous tissues it cannot be cholesterol itself, but some other substance far more potent in its effects than cholesterol.

The presence of cholesterol in brain tissue would in any case lead to alterations of the normal time relations of growth in animals to which the tissue was administered. To ascertain the possible effect of other components upon the growth of animals, therefore, it was necessary first of all to remove the cholesterol from the tissue. It was clearly recognized that this procedure might also result in the removal of the substance for which we were seeking. In that case, however, the search for such a substance would be narrowed down to the various products which are soluble in the agent (cold acetone) employed to extract the cholesterol. If, on the other hand, decholesterinized brain tissue were actually found to exercise a decisive influence upon the growth of animals, the existence of a potent catalyzer of growth in nervous tissue, other than cholesterol, would be thereby established. We accordingly undertook the investigation of the effects of decholesterinized brain tissue upon the growth of the white mouse.

Methods of Investigation.

The decholesterinized brain tissue was prepared by the method outlined by Rosenheim for the complete extraction of cholesterol from brain (18, 19). 5.5 kilos of minced ox brain were extracted with 5.5 liters of acetone at room temperature for 24 hours, the mixture being stirred from time to time. The fluid was then removed by filtration through several thicknesses of cheese-cloth, and the tissue squeezed between several layers of cheese-cloth to remove the greater part of the adhering liquid. The tissue was then again suspended in 5.5 liters of acetone, with occasional stirring, for 24 hours and the above process was repeated. In all, seven extractions were performed, the first three for 24 hours each, the last four for 12 hours each. We found that the last extract contained only inappreciable traces of cholesterol. Rosenheim reports that six extractions with cold acetone are sufficient to remove all the cholesterol from brain tissue.

The extracted tissue was dried by spreading it out in a thin layer upon glass plates and blowing a gentle current of air over it for 24 hours. It was then finely pulverized in a coffee mill and spread out to dry in still air for another 48 hours. The yield of dry decholesterinized tissue from 5.5 kilos of brain was 990 gm. Hence 1 kilo of fresh brain tissue corresponded to 180 gm. of the dry decholesterinized substance.

The daily dosage per mouse was arbitrarily fixed at 36 mg. It was felt that this large dose, corresponding to 200 mg. of fresh brain tissue, would certainly reveal the presence of any potent catalyzer of growth by decisive effects upon the time relations of growth. According to Rosenheim 1 kilo of fresh brain tissue contains 20 gm. of mixed cerebrosides (19). Hence 36 mg. contained 4 mg. of cerebrosides, a dose of this particular group of brain constituents equal to the dosage of tethelin which, in the experiments cited above, was found effective in causing marked departure from the normal growth curve in mice.

The animals employed in the experiment were taken, without selection, from litters 4 or 5 weeks old. Thirty-six males and the same number of females received the above mentioned dosage of brain tissue mixed with their daily supply of egg (5 cc. of mixed white and yolk for six mice). Since the growth curve of our normals had been previously found to alter from year to year (10) we simultaneously set aside thirty-six males and thirty-six females to serve as controls. So far as practicable the experimental and the control animals were alternately chosen from the same litters. Thus every litter containing four males, for example, contributed two to the experimental and two to the control group. The control animals received in every respect the same diet as the experimental animals with the exception that decholesterinized brain tissue was absent from the diet of the controls. The technique of the experiments and the items of the dietary were the same as in experiments previously described (20), with the exceptions that crushed oats were given instead of rolled barley, cabbage leaves twice a week in place of lettuce, and dry unsweetened biscuit ("Pilot bread") on Sundays in place of dried baker's bread. The animals were weighed weekly to within the nearest 0.5 gm.

The allowance of egg, whether containing brain tissue or not, was not completely eaten for the first 2 or 3 weeks. The amount of egg left unconsumed, however, was not greater in the one group of animals than in the other. After the first 3 weeks the egg was generally completely consumed each day. The decholesterinized brain tissue, with the exception of a few shreds of fibrous tissue, readily formed an emulsion when rubbed up with the egg, so that rejection of the tissue by animals which consumed the egg was impossible.

Experimental Results.

The results of the weighings from the 4th to the 13th week, when the experiment was terminated, are enumerated in Tables II and III. It will be seen that the deviation of the weights of the experimental animals from those of the control animals did not, in the case of the males, exceed the probable error of the estimate. The differences observed were therefore of a fortuitous character. In the case of the females somewhat greater variations were observed. Subsequently to the 10th week the variations from normality averaged about twice the probable error, the chance of their being fortuitous being about one in five. Subsequently to the 23rd week and until the 30th the observed variations were three times the probable error, the chance of a fortuitous origin being still as much as one in twenty (10).

When we compare these results with the consistent deviations from normality, amounting to five or six times the probable error, which are displayed by animals receiving 4 mg. of tethelin or 40 mg. of cholesterol daily (17), it is evident that the results obtained in this experiment do not afford any evidence for the existence of a growth catalyzer in decholesterinized brain tissue. The absolutely negative result obtained with the male animals may, in fact, be taken to disprove the presence of any such substance in the preparation administered to them.

Such variations from the weights of the control animals as were observed, however, were in the same sense in both males and females, the experimental animals being in each case slightly superior in weight to the control animals subsequently to the 10th week of age or 6th week of the experiment. This fact may possibly have some significance, but such significance as it possesses is probably quite unconnected with the presence of any specific growth hormone in the preparations administered. Such a slight difference might arise from no other source than the greater variety of constituents in the dietary of the animals which received the decholesterinized brain tissue.

TABLE II.

Age.	No. weighed.	Weight.	Deviation from normal of 1919.	Times probable error of difference.	Variability.
wks.		*gm.*	*gm.*		*per cent*
4	27	10.31	+0.41	0.8	23.7
5	36	11.65	+0.41	1.1	20.9
6	36	12.99	−0.11	0.3	20.5
7	36	14.68	+0.05	0.1	19.3
8	36	15.71	−0.35	0.8	17.8
9	36	16.53	−0.43	1.0	17.2
10	36	17.39	+0.13	0.3	16.8
11	36	18.03	+0.27	0.6	15.0
12	36	18.24	+0.12	0.3	15.8
13	36	19.00	+0.31	0.7	15.4
14	36	19.36	+0.43	0.9	14.6
15	36	19.90	+0.91	1.8	13.8
16	36	20.03	+0.47	0.9	15.4
17	36	20.76	+0.66	1.3	14.8
18	36	20.82	+0.45	0.9	14.3
19	36	21.25	+0.53	1.0	14.1
20	36	21.60	+0.48	0.9	13.4
21	36	21.82	+0.38	0.7	14.6
22	36	22.08	+0.66	1.2	14.4
23	35	21.86	+0.03	0.1	16.4
24	35	22.10	−0.04	0.1	13.5
25	34	22.76	+0.64	1.3	12.0
26	34	22.97	+0.52	1.0	11.5
27	34	22.88	+0.32	0.6	12.2
28	34	23.06	+0.26	0.5	11.6
29	34	23.41	+0.61	1.2	11.8
30	29	23.26	+0.43	0.7	12.4

TABLE III.

Age.	No. weighed.	Weight.	Deviation from normal of 1919.	Times probable error of difference.	Variability.
wks.		*gm.*	*gm.*		*per cent*
4	22	9.18	−0.53	1.1	26.7
5	35	10.79	−0.33	1.1	16.4
6	35	12.39	−0.11	0.3	14.3
7	35	13.63	−0.06	0.2	12.5
8	35	14.63	+0.18	0.5	12.9
9	35	15.41	+0.32	1.0	11.4
10	35	15.81	+0.12	0.4	12.2
11	35	16.66	+0.64	1.9	11.3
12	35	17.21	+0.64	1.9	11.0
13	35	17.64	+0.50	1.8	10.4
14	35	18.17	+0.84	2.4	11.0
15	35	18.51	+0.72	2.1	10.4
16	35	18.47	+0.43	1.2	11.0
17	35	18.93	+0.45	1.2	10.4
18	35	19.20	+0.84	2.3	10.7
19	35	19.61	+0.91	2.5	9.9
20	35	19.66	+0.68	1.7	10.4
21	35	20.10	+0.99	2.8	9.8
22	35	20.33	+0.95	2.5	9.7
23	35	20.30	+0.91	2.4	9.1
24	35	20.67	+1.04	2.7	9.1
25	35	20.67	+1.10	2.8	9.6
26	35	21.11	+1.38	3.4	9.6
27	35	21.14	+1.34	3.1	9.7
28	35	21.34	+1.50	3.4	10.8
29	35	21.16	+1.36	3.0	10.3
30	29	21.43	+1.48	3.1	11.0

Discussion of Results.

The absence of any decisive effect upon growth of as large a dose as 36 mg. per mouse per day of decholesterinized brain tissue may be taken to indicate either that brain tissue contains no growth catalyzer other than cholesterol, or that all the substances in brain tissue which are capable of influencing growth are contained, with the cholesterol, in the cold acetone extract.

The alternative still remains that the sought for growth hormone is actually present in the residue after acetone extraction but is not absorbed, either because it is of such a character as not to be transmissible through ordinary (non-nervous) tissues or because it is decomposed with destruction of its growth-influencing powers, by the action of the digestive juices. If, however, the substance were so difficultly transmissible through ordinary tissues as to render impossible its absorption from the digestive tract then neither could it be distributed from the nerve fibers through the cells and tissues which they innervate. On the other hand the growth-influencing substances with which we have hitherto become acquainted do not lose their power of affecting growth by exposure to the hydrolyzing enzymes of the stomach and upper intestine.

The decholesterinized brain tissue which was employed in these experiments still contained, practically without diminution, all the phosphatides and cerebrosides of nervous tissue. The negative result obtained with this preparation therefore stands in sharp contrast to our positive results previously obtained with crude lecithin from eggs (21). While the dosage of lecithin employed in our previous experiments was larger than in the present experiments, we are inclined, in the light of the above results, to attribute our positive findings in the case of egg lecithin to incomplete purification, and probably to incomplete removal of acetone-soluble substances.

SUMMARY.

1. The nervous system can be shown (*a*) by its importance for the regeneration of lost parts, (*b*) by the significance of the ratio of brain weight to body weight in determining the longevity of animals, and (*c*) by the dystrophy following upon nerve section, to exert a stimulating influence upon the growth of parenchymatous tissues.

2. The stimulation of growth by nervous tissues may conceivably be due either to the nervous impulses conducted by them or to a substance officiating as a growth hormone which is conducted to the tissues wholly or in part through nerve fibers.

3. In conformity with the latter view it is shown that substances which stimulate the growth of parenchyma in mice so modify the time relationships of growth as to bring about an approximation of the growth curve for mice towards the form of

the growth curve for human beings, in which the ratio of brain weight to body weight is much higher than it is in mice.

4. Cholesterol has previously been shown to modify the growth of mice in the manner indicated. Its effect, even in high dosage, is, however, much less than the effect of a high ratio of brain weight to body weight. If the nervous system affects growth through the agency of a specific catalyzer or hormone, therefore, this substance must be much more potent than cholesterol.

5. It is shown that brain tissue from which the cholesterol has been extracted by cold acetone, administered by mouth in dosage of 36 mg. daily, is without effect upon the growth of mice.

6. It is inferred that if nervous tissues do contain a potent catalyzer of growth other than cholesterol it is also soluble in cold acetone and is removed from the brain in the process of extracting the cholesterol.

BIBLIOGRAPHY.

1. Child, C. M., Senescence and rejuvenescence, Chicago, 1915, 206.
2. Loeb, J., *Arch. ges. Physiol.*, 1894, lvi, 247.
3. Morgan, T. H., Regeneration, New York and London, 1901, 52.
4. Herbst, C., *Arch. Entwcklngsmechn. Organ.*, 1899–1900, ix, 215; 1901–02, xiii, 436.
5. Friedenthal, H., *Zentr. Physiol.*, 1910–11, xxiv, 321.
6. Dreyer, G., Ray, W., and Walker, E. W. A., *Proc. Roy. Soc. London, Series B*, 1912–13, lxxxvi, 39, 56. Dreyer, G., and Walker, E. W. A., *Proc. Roy. Soc. London, Series B*, 1913–14, lxxxvii, 319.
7. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 427.
8. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1920, xlii, 71.
9. Quetelet, L. A. J., Anthropométrie, cited in the Report of the British Anthropometric Committee, British Association Report, 1879, 175.
10. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 377.
11. Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1907–08, xxv, 581; *Am. J. Physiol.*, 1915, xxxvii, 1; *J. Biol. Chem.*, 1916, xxiv, 363.
12. Chalatow, S. S., *Virchows Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. Path.*, 1914, lvii, 85. Anitschkow, N., *Beitr. path. Anat. u. allg. Path.*, 1913, lvi, 379; 1914, lvii, 201. Bailey, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 68; 1915–16, xiii, 60. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 443.
13. Loeb, J., The dynamics of living matter, New York, 1906, 216.
14. Meyer, H., and Ransom, F., *Arch. exp. Path. u. Pharmakol.*, 1902–03, xlix, 369.

15. Dorée, C., and Gardner, J. A., *Proc. Roy. Soc. London, Series B*, 1908, lxxx, 227. Ellis, G. W., and Gardner, J. A., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 129; 1911–12, lxxxiv, 461.
16. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxv, 635.
17. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 393.
18. Rosenheim, O., *J. Physiol.*, 1906, xxxiv, 104.
19. Rosenheim, O., *Biochem. J.*, 1913, vii, 604.
20. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347.
21. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxv, 647.

THE DIASTATIC FERMENTS OF THE BLOOD.

By D. SCLATER LEWIS and EDWARD H. MASON.

(*From the Metabolism Clinic, the Royal Victoria Hospital, Montreal, Canada.*)

(Received for publication, September 9, 1920.)

The "isolation" of the diastatic ferments dates back to Payen and Persoz (1) who in 1833 precipitated a substance called diastase from extract of malt. In 1846 Magendie (2) drew attention to the diastatic properties of the blood, and showed that blood was able to split starch to dextrin and glucose. Since that time an enormous literature has grown up dealing with almost every phase of diastatic activity. Most of these older writings refer to work on the experimental animals, Foster (3) being one of the few whose studies refer to the diastases in diabetes. He reports a diminished excretion of the ferment in diabetic urine, and he also emphasizes the fact that this starch-splitting power of the urine is not due to any bacterial growth, but results from the action of a specific amylolytic ferment.

Site of Origin of the Ferment in the Body.

The diastases have such a widespread distribution throughout the body that there is some doubt as to where they are actually produced. They are found in the cells of the pancreas, the salivary glands, the liver, blood, lymph, muscles, and the kidneys (4). Schlesinger (5) believes that most of the tissue diastases are formed by the pancreas and distributed throughout the body by means of the blood stream. The reaction of the blood diastases to a complete removal of the pancreas is of interest in this regard. Immediately after the operation there is a sharp drop in the diastase, as if the principal source of supply was cut off, but within 4 or 5 days the ferment returns to normal levels. This points to the production of the ferment by the tissues at large, as well as by the pancreas, although the latter may well be the principal source of supply under normal conditions.

Rôle of the Diastase in the Blood.

A question closely allied to the above is whether the blood diastase has any importance in the production of the hyperglycemia of diabetes and nephritis, or whether it is merely a substance discarded by the cells and on its way to the excretory organs. It is generally believed that there is an increased breaking down of glycogen to dextrose in diabetes, but Milne and Peters (6) think this is due to the accumulation of some substance in the body, which stimulates the glycogenolytic activity of the cells; they do not think the blood diastase is augmented to a sufficient extent to account for this change. They find no increased diastatic activity in phlorhizin glycosuria, and other writers state that piqûre causes no change in the diastatic activity of the blood, although under both of these conditions there is a tremendous mobilization of sugar. Bang (7) states that there is no relation whatever between the blood diastase and the degree of glycogenolysis and glycosuria. Frequently he finds a marked glycosuria with a low blood diastase, and Bainbridge and Beddard (8) also report two cases of severe diabetes with an almost complete absence of diastase from the blood. Myers and Killian (9), however, report a series of cases of diabetes in which they find a close relation between the level of the blood diastase and the severity of the diabetes. In view of the interesting character of their results, we decided to repeat their work, making use of a modified form of their method of estimating the diastatic activity. This modification consisted in using the Benedict (10) method of blood sugar determination instead of that described by Myers and Bailey.

Method.

Two 2 cc. samples of blood are placed in 25 cc. volumetric flasks. The first is laked with 4 cc. of distilled water and used as the control, while only 3 cc. of water are added to the second flask. Both flasks are placed in a water bath at 40°C., and when the temperature of the flasks has become constant 1 cc. of a 1 per cent solution of soluble starch is added to the second flask, and the incubation is then carried on for 15 minutes. Both flasks are then cooled, made up to the mark with the sodium picrate-picric acid solution, and the sugar is estimated according to Benedict. The quantity of starch which has been broken down by the diastase is calculated by comparing the amounts of sugar in the control with that in the blood-starch mixture.[1] The index is the percentage of soluble starch which has been converted into dextrose. For example, if 2 mg. of starch are changed to sugar, the index will be 20. All tests in the series have been done on duplicate samples within 15 minutes of the withdrawal of the blood. Our results for normal subjects are in close agreement with those of Myers and Killian (9) and of de Niord and Schreiner (11), but there is a wide divergence in the indices of the diabetic patients. To exclude the modified technique as a possible cause of this difference, the original method has been compared with the one used for this series and the results have been found to be almost identical by the two methods (Table I).

TABLE I.

Comparison of Indices by Original and Modified Methods.

Case No.	Diastatic index.		Diagnosis.
	Modified method.	Original method.	
1	23.4	21.2	Laboratory worker.
2	13.6	13.0	" "
3	10.4	12.2	" "
4	16.6	15.6	" "
5	18.7	19.4	" "
6	22.2	25.0	Diabetes.
7	11.4	11.4	"
8	33.2	32.4	Nephritis.

Normal Subjects.—The normal subjects consist of workers in the laboratory, students, and convalescent patients in the wards. In all there are studies on thirteen persons and a total of 58 determinations of the normal diastatic index. There is a considerable variation in the index, namely from 7.0 to 29.4, but 47 of the 58 are within the comparatively limited range of 14 to 25.

---

[1] A small correction factor is necessary for the amount of sugar present in the soluble starch (Lintner). This usually amounts to 0.25 mg. per 10 mg. of starch, or a correction factor of 2.5 points in terms of the index.

These then may be taken as the usual limits of the normal index. Most of the examinations were made on fasting bloods, but, as a number had been done on blood taken after food, it seemed desirable to study the diastatic activity in relation to food and to determine whether or not food or the type of food had any effect on the blood diastase. For this purpose a group of five normal individuals were each given a series of test breakfasts. These meals were so arranged that the first was an ordinary breakfast, the second contained a large proportion of carbohydrate, the third a high protein content, and the fourth a large excess of fat. The actual composition of these meals is given in Table II. A specimen of blood was taken while the subject was fasting, and a second specimen was withdrawn 1½ hours after the middle of the meal. The diastatic index was estimated for these two samples of blood. The average indices before and after food are given in Table II. These results do not show any marked change in the index after these test diets. As a general rule, the index is lower after meals than before, but the average depression is only 1.2 points after the ordinary meal, 2.0 points after the protein, and 2.52 after the fat meal. After the carbohydrate meal the index shows a rise in four of the five tests, but even here the variation is only 2.1 points. The diastatic index evidently undergoes very slight changes in relation to the various types of food. This agrees with Watanabe's (12) results on experimental animals and also with the work of earlier writers.

TABLE II.

Type of meal.	Protein.	Fat.	Carbohydrate.	Average. Index before breakfast.	Average. Index after breakfast.	Difference.
	gm.	*gm.*	*gm.*			
Ordinary breakfast.	(?)	(?)	(?)	18.5	17.3	−1.2
Carbohydrate "	31.0	32.0	142.0	17.0	19.1	+2.1
Protein "	48.0	8.0	10.0	20.8	18.8	−2.0
Fat "	13.0	66.0	32.0	18.76	16.24	−2.52

Cases of Diabetes.—The chief interest naturally is attached to the series of diabetics. These are eighteen in number and represent all stages of the disease. As stated above, Myers and

Killian (9) report an almost constantly increased diastatic activity in these cases, and this increase is more or less proportional to the level of the blood sugar and to the severity of the diabetes. De Niord and Schreiner (11) confirm these findings except for a series of diabetics with syphilis in whom they find extremely low indices. These results are not borne out by the present series of studies where the index is consistently lower than normal. Only six of the twenty-seven are above 15, while the index is 10 or lower in fourteen instances (normal range 14 to 25). Table III is arranged according to the level of the blood sugar and there is no obvious relation between the level of the sugar in the blood and the degree of diastatic activity. Two or more determinations were done on five of the cases. In three (L., McL., and McD.) the index is more or less proportional to the level of the blood sugar; *i.e.*, a high sugar is associated with a high diastatic index. In two cases (Ch. and R.), however, the index is inversely proportional to the level of the sugar in the blood. These two values are therefore not always proportional to each other.

These results are in such marked contrast to those reported by Myers and Killian (9) that a possible explanation was sought for. De Niord and Schreiner (11) mention syphilis as a cause of a low diastatic index but no case in the series gave a positive Wassermann. It was thought that possibly the age of the patient and the duration of the disease might have some relation to the height of the index. Chronic nephritis is notoriously frequent in old diabetics and it was possible that the high indices might have been due rather to a retention of the diastase by a damaged kidney, than to any particular change in the rate of production of the ferment in the diabetic individual. Reference to Table III will show that there is a marked tendency to the lower indices in the very young, the highest index in those below 13 years being 13.8. On the other hand L., a woman of 63 years, and a diabetic of 16 years standing, had on three occasions the very low values of 10.4, 8.6, and 4.0. Therefore age and duration of the disease cannot be important factors in determining the level of the diastatic activity. In the present series a low index has not been a favorable sign, as all the fatal cases, three in number, had indices below 11.5.

In the present state of our knowledge it seems reasonable to believe that the percentage concentration of the blood diastase has very little direct relation to the rate at which starch is broken down by the tissues of the diabetic organism, and it is quite possible that Carlson and Luckhardt (13), King (14), and others are correct in regarding the diastatic bodies of the blood as discarded elements on their way to excretion by the kidneys.

TABLE III.

Diastatic Index in Diabetes.

Case.	Date.	Age.	Blood sugar per 100 cc.	Index.
	1920	*yrs.*	*mg.*	
J.		32	424	8.4
S.		53	384	10.0
Ch.*	Apr. 13	39	360	8.4
	" 21		280	11.4
Z.*		6	356	3.6
L.*	Apr. 13	63	352	10.4
	" 13		384	8.6
	" 15		314	4.0
A.		9	314	6.2
D.		62	302	22.2
Ro.		63	302	18.4
	1919			
McL.	Nov. 19	23	300	15.2
	" 28		262	6.4
	Dec. 26		194	10.0
P.		11	260	11.4
F.		42	230	4.8
McD.	Dec. 29	37	224	7.6
	1920			
	Feb. 11		266	22.2
	" 16		240	16.2
	1919			
R.	Nov. 19	34	222	2.0
	Dec. 18		187	9.8
	1920			
	Feb. 13		145	14.6
C.		64	202	7.3
H.		12	189	13.8
Rd.		11	178	12.2
U.		64	150	8.0
M.		74	117	22.0

* Died.

Cases of Nephritis.—In 1909 Wohlgemuth (15) introduced the determination of the urinary diastase as a test of renal function, but Rowntree, Marshall, and Baetjer (16) found the test unreliable and it received very little credence in this country, although it is still used to some extent in England. More recent studies of the blood diastase in nephritis have shown that this test has no great reliability in measuring renal function. The index may be high or low with a disease of the same degree of severity and apparently of the same type. In the present series for example, Ch. and Vi. are both cases of severe diffuse nephritis with extreme anasarca, but with relatively little disturbance in the rate of

TABLE IV.

Cases of Nephritis.

Case.	Date.	Blood sugar per 100 cc.	Diastatic index.	Ambard K.	Blood urea per liter.	Albuminuria.	Diagnosis.
	1920	*mg.*			*gm.*		
Ch.	June 8	123	18.2	0.139	0.510	+++	Chronic diffuse nephritis. Edema.
Vi.	Jan. 23	120	5.2	0.177	0.300	+++	" " "
	Feb. 10	126	6.6	0.123	0.204	+++	
	Apr. 29	134	9.4	0.077	0.195	++	
Sm.		148	4.0	0.078	0.360	+	Acute nephritis.
Bu.	Feb. 19	120	33.2	0.162	0.735	++	" "
	" 19	168	25.2				
	1919						
Da.	Dec. 1	127	20.0	0.141	0.585	++	" "
	1920						
	Apr. 23	158	37.8	0.247	0.630	+	" "
Mo.		122	14.0	0.082	0.150	+	Chronic diffuse nephritis.
La.		200	46.0	0.058	0.150	+	" " "
Ak.		136	28.2	0.570	1.275	++	Secondary contracted kidney.
Dr.	Apr. 27	122	12.0	0.168	0.270	Trace.	" " "
	May 4	102	17.8	0.089	0.360	"	
Sa.	Apr. 14	137	26.0	0.158	0.555	+	Primary contracted kidney.
	" 27	125	8.0	0.147	0.600	Trace.	
Sc.		144	8.0	0.117	0.436	"	" " "
Mu.		107	23.6	0.070	0.270	"	Cardiorenal disease.

nitrogen excretion (Ambard: 0.139 for Ch. and 0.177 for Vi.), Ch. gives a normal diastatic activity, while in Vi. there is a very low index which shows no change even with marked improvement in the patient's condition. Again, Sa. shows a change from an index of 26 to one of 8.0 within a fortnight without the other tests of function showing any noticeable variation and without any change in his clinical condition. Again, Ak., who died with extreme renal insufficiency 2 weeks after the diastatic test, showed no greater retention than either Da. or Bu. both of whom are alive and show no marked clinical evidence of renal insufficiency.

The diastase of the blood shows wide variations in the blood of nephritic patients; in some cases it is in accord with other tests showing a retention of waste products, but in a considerable number of cases studied it shows no direct relation to the type of renal lesion or to the degree of involvement or progress of the disease.

SUMMARY.

1. The normal diastatic index is between 14 and 25.
2. The diastatic activity of the blood shows very little change in relation to food. This applies both to ordinary meals and to those in which there is a large preponderance of one particular type of food.
3. There is no constant increase in the blood diastase in diabetes and there does not seem to be any direct relation between the height of the blood sugar and the diastatic activity. Some of the most severe cases show the lowest diastatic activity.
4. There is a marked variation in the level of the ferments in cases of nephritis, but no evident connection between the type of disease and the diastatic activity, and no constant relation between the progress of the lesion and the diastatic index.

We are indebted to Miss Lane, the technical assistant, for many of the blood sugar determinations.

BIBLIOGRAPHY.

1. Payen and Persoz, *Ann. chim. phys.*, 1833, liii, 73, cited by Bayliss, W. M., Principles of general physiology, London, New York, Bombay, Calcutta, and Madras, 1915, 307.
2. Magendie, *Compt. rend. Acad.*, 1846, xxiii, 189.
3. Foster, M., *J. Anat. and Physiol.*, 1867, i, 107.
4. Bial, M., *Arch. ges. Physiol.*, 1892, lii, 137. Hamburger, C., *Arch. ges. Physiol.*, 1895, lx, 543. Borchardt, L., *Arch. ges. Physiol.*, 1903, c, 259.
5. Schlesinger, W., *Deutsch. med. Woch.*, 1908, xxxiv, 593.
6. Milne, L. S., and Peters, H. le B., *J. Med. Research*, 1912, xxvi, 415.
7. Bang, I., cited by Schlesinger (5).
8. Bainbridge, F. A., and Beddard, A. P., *Biochem. J.*, 1907, ii, 89.
9. Myers, V. C., and Killian, J. A., *J. Biol. Chem.*, 1917, xxix, 179.
10. Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203.
11. de Niord, H. H., and Schreiner, B. F., *Arch. Int. Med.*, 1919, xxiii, 484.
12. Watanabe, C. K., *Am. J. Physiol.*, 1917–18, xlv, 30.
13. Carlson, A. J., and Luckhardt, A. B., *Am. J. Physiol.*, 1908–09, xxiii, 148.
14. King, C. E., *Am. J. Physiol.*, 1914, xxxv, 301.
15. Wohlgemuth, J., *Biochem. Z.*, 1909, xxi, 432.
16. Rowntree, L. G., Marshall, E. K., Jr., and Baetjer, W. A., *Arch. Int. Med.*, 1915, xv, 543.

CERTAIN FACTORS THAT INFLUENCE ACETONE PRODUCTION BY BACILLUS ACETOETHYLICUM.*

By C. F. ARZBERGER, W. H. PETERSON, and E. B. FRED.

(From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.)

(Received for publication, July 3, 1920.)

INTRODUCTION.

The production of acetone by the lower organisms has formed the subject of a number of recent investigations. During the war the great demand for acetone and allied solvents of nitrocellulose and of "aeroplane dopes" stimulated the development of processes for the production of acetone on a commercial scale.

Schardinger (1905) isolated an organism which fermented carbohydrates with the formation of ethyl alcohol, acetone, and acetic and formic acids. The majority of the carbohydrates were fermented by this organism with the formation of these products. Potatoes and potato starch proved the best media, and in the presence of peptone and calcium carbonate acetone equal to 6.9 per cent of the starch, and alcohol equal to 20.8 per cent by weight were found. Acetic and formic acids were also produced, the latter predominating.

The demand for butyl alcohol and acetone in the manufacture of synthetic rubber from butadiene and isoprene stimulated further research for an organism that would produce both of these compounds in quantities to be of commercial value. Fernbach and Strange (1912) isolated an organism of this type. Weizmann (1915), a student of Fernbach's, subsequently isolated a heat-resisting organism, *Bacillus granulobacter pectinovorum*, from soils and cereals which had the characteristic ability to convert starch mainly into acetone and butyl alcohol. This organism was then used to produce acetone on a commercial scale by fermenting mashes of damaged rice, grain, and horse chestnuts. A factory was successfully operated from January, 1917, to November, 1918, with the production of about 200 long tons monthly. In an extensive study of the biochemistry of this process Speakman (1920) reported the production of 5,741,273 pounds of acetone, and 12,660,834 pounds of alcohol, or 7.76 per cent acetone and 17.23 per cent alcohol of the total 73,463,654 pounds of dry corn fermented.

---

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station. This work was in part supported by a grant from the special research fund of the University of Wisconsin.

Northrop, Ashe, and Senior (1919) succeeded in isolating an organism, *Bacillus acetoethylicum*, from old potatoes which possessed certain characteristics similar to those of Schardinger's organism in that it converts most carbohydrates into ethyl alcohol, formic acid, and acetone. The amount was about 8 to 9 per cent acetone and 14 to 20 per cent alcohol by weight of the original carbohydrate. The work of Northrop and his collaborators showed that the optimum temperature for growth and production of acetone was about 43°C. For the maximum production of acetone the reaction of the medium must be about pH 6.0 to 7.0; an excess of calcium carbonate was added to maintain this reaction. This hydrogen ion concentration of the medium for acetone production was found to be very different from that required for optimum growth; namely, pH 8.0 to 9.0. It was noted that if the medium was first adjusted to the above reaction a vigorous fermentation took place. This fact was later made use of in the plant of the Commercial Solvents Corporation at Terre Haute, Indiana, where experiments to produce acetone commercially were carried out. Before the mash was run into the fermenters, it was brought to pH 8.5 to 9.5 by the addition of lime and it was found that the fermentation was similar to that obtained in the laboratory experiments.

Reilly and his collaborators (1920) studied in detail the mechanism of the fermentation process for the production of acetone and butyl alcohol from carbohydrate material. In the initial stage of fermentation the acidity increased rapidly until the maximum was attained. After this point was reached, there was a marked gain in the rate of production of acetone, butyl alcohol, carbon dioxide, and hydrogen. These investigators suggested that acetic and butyric acids were intermediate products in the formation of acetone and butyl alcohol. In the presence of calcium carbonate, they found that the production of acetone and alcohol was almost entirely suppressed. When acetic acid was added to the fermenting culture, there was an increased yield of acetone without any change in yield of butyl alcohol.

In this paper a study has been made of the products formed, the factors influencing the end-products, and the relation of these products to one another in the production of acetone by *Bacillus acetoethylicum*.[1]

---

[1] Dr. John H. Northrop of The Rockefeller Institute for Medical Research kindly placed at our disposal, for this work, a pure culture of *Bacillus acetoethylicum*, and we wish to express our thanks to him for this courtesy.

EXPERIMENTAL.

In the fermentation of glucose, sucrose, potato starch, and xylose, 2 per cent solutions were used. The medium consisted of 0.5 per cent peptone, 0.1 per cent dipotassium phosphate, tap water, and the desired carbohydrate. Of this solution 250 cc. were placed in 300 cc. Erlenmeyer flasks, and the flasks sterilized for 30 minutes at 15 pounds pressure. The flasks were next inoculated with *Bacillus acetoethylicum;* to some of these flasks $CaCO_3$ was added, to others 2 or 3 drops of brom-cresol purple were added, and the medium was adjusted to the desired pH by adding NaOH. In certain experiments the flasks were connected to a carbon dioxide absorption trap similar to that used by Peterson and Fred (1920), and incubated at 37°C.

The acids formed during the fermentation were neutralized daily by additions of sterilized 1 N sodium hydroxide, except in the cultures to which an excess of sterilized calcium carbonate was added at the time of inoculation. Here, as with the other cultures, the progress of the fermentation was indicated by the rapid evolution of gas and frothing of the cultures. In some cases further additions of sterilized calcium carbonate were found necessary. At the end of the fermentation period, the duration of which was approximately 2 weeks, the cultures were analyzed for volatile and non-volatile acids, alcohol, acetone, carbon dioxide, and unfermented carbohydrates.

Volatile and Non-Volatile Acids.—The determination of these acids was made in the same manner as has already been described in previous publications.

Alcohol and Acetone.—To 75 or 100 cc. of the culture solution in a Kjeldahl flask, 40 gm. of C. P. sodium chloride were added and from two-fifths to one-half of the original volume was distilled over. The distillate was made up to 100 cc., 50 cc. of which were used for the alcohol determination and 50 cc. reserved for the determination of acetone. The alcohol was determined by oxidizing and distilling a 50 cc. aliquot according to the method of Dox and Lamb (1916). After titration with 0.1 N barium hydroxide, this distillate was evaporated to dryness on the steam bath and the residue used for the Duclaux determination of acids.

Acetone.—The remaining 50 cc. of the alcoholic acetone solution were used for determining the amount of acetone in the cultures by Goodwin's (1920) modification of Messinger's method. A 10 cc. aliquot of the distillate was pipetted into a glass-stoppered bottle, 10 cc. of a 10 per cent solution of sodium hydroxide were added, and an excess of 0.1 N iodine solution was run in with constant agitation. The bottle was then stoppered and allowed to stand 10 minutes, after which 11 cc. of 2 N sulfuric acid were added, and the excess of iodine was titrated with 0.1 N sodium thiosulfate.

Carbon Dioxide.—The carbon dioxide in the culture and in the potassium hydroxide of the absorption bottle was determined by means of the Van Slyke (1917) apparatus for determining carbon dioxide in blood and carbonate solutions.

Unfermented Carbohydrates.—The unfermented carbohydrates in the culture were determined by the Brown, Morris, and Millar (1897) method. The starch and sucrose were hydrolyzed with hydrochloric acid, then clarified with subacetate of lead, and aliquots taken for gravimetric analysis.

The results of the analysis of the glucose, sucrose, potato starch, and xylose cultures are given in Table I. The figures show the two different types of fermentation obtained by titrating the acid produced daily with 1 N sodium hydroxide, and by keeping the alkalinity constant with an excess of calcium carbonate. In the sodium hydroxide series there is formed a large amount of volatile acid and a relatively small amount of alcohol and acetone. On the other hand the calcium carbonate series shows an increase in the alcohol and acetone production with a corresponding decrease in the volatile acid. The non-volatile acid is practically the same in both series. The data of the table indicate that the optimum conditions for the production of acetone are maintained in cases where calcium carbonate is used to neutralize the acid formed during fermentation. Northrop states that when calcium carbonate is used the medium is maintained at pH 6.0 to 7.0. If the medium is more alkaline and near the optimum for the growth of *Bacillus acetoethylicum*, the organism multiplies rapidly and at the same time produces sufficient acid to change the reaction to about pH 6.0 to 7.0 where it is maintained by the calcium carbonate. The pH of some of the cultures which had been fermenting for 4 days in the presence of an excess of calcium carbonate was measured and it was found that the reaction was pH 5.6 to 5.8.

An interesting fact noticeable in the fermentation, where the acid formed was titrated daily with 1 N sodium hydroxide, was the destruction of the indicators. Brom-cresol purple and cresol red were used, and after 24 hours of vigorous fermentation it was found that the indicators had been reduced. To insure the proper reaction, additions of sterilized indicator were made before each titration.

TABLE I.

Products Formed in the Fermentation of Various Carbohydrates.
Calculated for 100 Cc. of Cultures.

Carbohydrate.	Reaction.	Neutralizing substance.	Volatile acid as acetic.	Non-volatile acid as lactic.	Ethyl alcohol.	Acetone.	Carbon dioxide.	Unfermented carbohydrate.	Total.
	pH		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1. Glucose...	6.2	NaOH	0.3192	0.0684	0.2723	0.0986	0.3634	0.8696	1.9915
2. " ...	5.8	$CaCO_3$	0.0869	0.0522	0.3202	0.1464		0.6060	1.2117
3. Sucrose....	6.2	NaOH	0.2136	0.0522	0.1600	0.0399	0.2517	1.0024*	1.7198
4. "	5.8	$CaCO_3$	0.1410	0.0635	0.4554	0.2002		0.1789	1.0390
5. Starch, potato...	6.2	NaOH	0.3732	0.0504	0.2972	0.0733	0.3785	0.3423	1.5149
6. Starch, potato...	5.8	$CaCO_3$	0.0704	0.0632	0.4329	0.2034		0.3845	1.1544
7. Xylose....	6.2	NaOH	0.3480	0.0657	0.4425	0.1568	0.5346	0.2218	1.7614

* Fermentation incomplete.

Identification of Volatile Acids.—The dry residue of the barium salts of the volatile acid was taken up with water and filtered to remove the barium carbonate. The filtrate was then made up to 80 cc. and treated with the proper amount of 1 N sulfuric acid to liberate the volatile acids. The barium sulfate formed was filtered off, and the filtrate was made up to 110 cc. and distilled according to the Duclaux procedure. The distilling constants obtained (Tables II and III) indicate the presence of both acetic and formic acids. The approximate percentages of formic and acetic acids in the above cultures were computed according to the graphic method of Gillespie and Walters (1917) with results as given in Table IV. The results of this table indicate that acetic acid predominates in the fermentation of sucrose and potato starch, whereas formic acid is produced more abundantly in the fermentation of xylose.

TABLE II.

Volatile Acids from the Fermentation of Sucrose as Determined by the Duclaux Method.

	Fraction.									
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Individual fraction, *0.1* N *cc*..	1.57	1.72	1.82	1.96	2.03	2.19	2.38	2.70	3.14	4.10
Total, *0.1* N *cc*...	1.57	3.29	5.11	7.07	9.10	11.29	13.67	16.37	19.51	23.61
Distilling constant found....	6.6	13.5	21.6	29.9	38.5	47.8	57.8	69.3	82.6	100.00
Distilling constant calculated*.........	6.79	13.9	21.6	29.7	38.2	47.6	57.6	69.0	82.5	

* Formic 40.55, acetic 59.55 (see Table IV).

TABLE III.

Distilling Constants of Volatile Acids Obtained by the Duclaux Method.

	Fraction.									
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Glucose....................	7.0	14.6	22.6	31.0	39.9	49.7	60.0	71.6	84.4	100
Potato starch..............	6.8	14.0	21.6	29.5	38.1	47.3	57.4	68.7	81.8	100
Xylose.....................	6.5	13.4	20.9	28.7	37.2	46.2	56.4	67.9	81.8	100

TABLE IV.

Percentage Composition of the Volatile Acids from the Sodium Hydroxide Series.

Culture.	Formic.	Acetic.
	per cent	*per cent*
Sucrose..................................	40.55	59.55
Potato starch............................	43.32	56.68
Xylose...................................	54.04	45.96

A quantitative estimation of the formic acid from the residue of the Duclaux distillation was made according to the method of Fincke (1913) and substantially the same results were obtained as from the Duclaux distillation.

Identification of Non-Volatile Acids.—The dry residue obtained by evaporating the titrated solution of non-volatile acid was taken up in water, filtered to free it from any $BaCO_3$, and again evaporated to dryness on a steam bath in a tared platinum dish. The platinum dish and residue were dried in an electric oven at 130°C. until constant weight was obtained, an excess of dilute sulfuric acid was added, and the salts were converted into barium sulfate by ignition, and then weighed. From the weight of the dried barium salts the theoretical weight of barium sulfate was calculated. The slight discrepancy between the found and calculated values is probably due to ether-soluble decomposition products formed during the sterilization of the sugars. The results of this work are given in Table V.

TABLE V.

Composition of Barium Salts of Non-Volatile Acids.

Carbohydrate.	Weight of barium salt.	Weight of barium sulfate.	
		Found.	Calculated for $Ba(C_3H_5O_3)_2$.
	gm.	*gm.*	*gm.*
Glucose	0.1121	0.0770	0.0830
Potato starch	0.0936	0.0678	0.0692

TABLE VI.

Distilling Constants of Alcohols of NaOH Series by Duclaux Method.

	Fraction.									
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Sucrose	7.7	15.5	23.9	32.4	41.0	51.4	61.6	72.8	85.3	100
Potato starch	7.7	15.8	24.4	33.0	42.3	51.9	62.1	73.0	85.4	100
Xylose	7.7	15.5	24.1	33.0	42.3	51.6	61.8	73.0	86.0	100
Glucose	7.8	15.9	24.3	33.1	42.3	51.9	62.2	73.2	85.6	100
Sucrose*	7.7	15.7	24.2	32.9	42.2	51.7	62.0	73.1	85.5	100
Potato starch*	7.7	15.8	24.2	32.8	41.9	51.3	61.5	72.6	85.1	100
Constants for acetic acid	7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100

* $CaCO_3$ series.

Identification of Alcohols.—The solution of the barium salts obtained by oxidation of the alcohol was evaporated to dryness on a steam bath and prepared for the Duclaux distillation in a way similar to that used for the volatile acids. The results obtained are given in Table VI. The Duclaux constants in this table show that the alcohol produced during fermentation is ethyl and that only traces of any higher alcohols are present. Northrop, Ashe, and Senior report that ethyl, propyl, and butyl alcohols are produced.

Modification of the Products of Fermentation by Varying the Reaction of the Medium.

It has already been noted that the reaction of the medium influences the products of fermentation but, so far, the experiments have been limited to only two reactions, pH 5.8 to 6.2. Further tests of this point were made in which 2 per cent sugar solutions containing varying amounts of 1 N NaOH or calcium carbonate were fermented. No attempt was made to maintain constant the reaction of the medium by the use of a high concentration of buffer substances.

7.5 gm. of peptone and 1.5 gm. of K_2HPO_4 were dissolved in 1.5 liters of tap water. Portions of 250 cc. of this medium were then pipetted into 300 cc. Erlenmeyer flasks, and 5 gm. of either glucose or sucrose were added to each flask. After sterilization one flask each of glucose and sucrose was inoculated and an excess of $CaCO_3$ added. A second pair of glucose and sucrose flasks was inoculated, 4 drops of sterilized brom-cresol purple were added, and the pH of the medium was adjusted to the indicator by adding sterilized 1 N NaOH. To a third pair of flasks, 2 cc. of sterilized cresol red were added and the medium was similarly adjusted with NaOH. These last four flasks were closed with two-hole stoppers. Through one hole was inserted a short piece of glass tubing, to the upper end of which was fastened a piece of rubber tubing plugged with cotton and closed with a screw-clamp. Through the second hole was passed a piece of glass tubing bent at an angle of about 60° and drawn out to a capillary point. The flasks were incubated at 37°C. and sterilized 1 N NaOH and indicator were added as the fermentation progressed. At the end of 14 days the cultures were removed from the incubator and analyzed. The results given in Table VII show the effect of varying the hydrogen ion concentration on the volatile acid,

TABLE VII.

Comparison of Products Obtained under Various Reactions of Media. Calculated for 100 Cc. of Culture.

Carbohydrate.	Reaction.	Neutralising substance.	Volatile acid as acetic.	Ethyl alcohol.	Acetone.	Unfermented carbohydrates.
	pH		*gm.*	*gm.*	*gm.*	*gm.*
Glucose	5.8	$CaCO_3$	0.0720	0.4117	0.1359	0.2296
"	6.2*	NaOH	0.3276	0.3128	0.0685	0.3492
"	8.0†	NaOH	0.5040	0.2088	0.0995	0.3235
Sucrose	5.8	$CaCO_3$	0.2556	0.4752	0.1064	0.4865
"	6.2	NaOH	0.4404	0.5060	0.1531	0.4606
"	8.0	NaOH	0.5400	0.3468	0.1207	0.5074

* Brom-cresol purple.
† Cresol red.

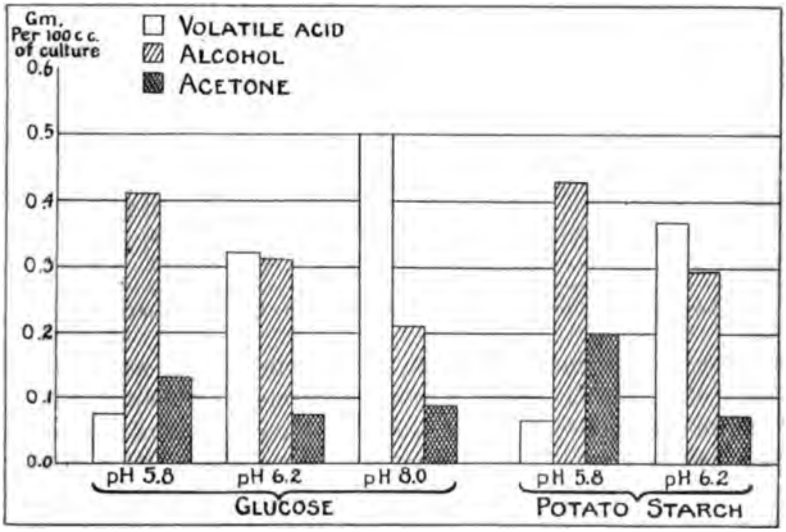


Fig. 1. The influence of varying the reactions of the medium on the products of fermentation.

acetone, and alcohol production. The data from the $CaCO_3$ cultures are comparable to those obtained in the first experiment with the exception that the fermentation was more nearly complete. An increase in the pH of the medium results in an increase in volatile acid and a decrease in alcohol production. The non-volatile acid remains practically constant and in amounts approximating those in Table I. A graphic representation of the change of products brought about by varying the pH is given for the two carbohydrates, glucose and starch. Both the table and Fig. 1 show the pronounced influence of the pH of the media in determining the relative amounts of the products formed. In a second parallel experiment, $CaCO_3$ was omitted and the different reactions were obtained by means of sodium hydroxide only. The cultures were adjusted to pH values of 5.5, 6.5, 7.5, and 8.5, inoculated, and after 10 days incubation were analyzed. The culture with pH 5.5 did not ferment very readily as indicated by the results of the analysis in Table VIII. The percentage of the total products, however, shows the same decrease of alcohol and acetone production with an increase in the pH as given in Table VII. From these two sets of experiments which in general confirm each other, it will be seen that the products of fermentation are to a great degree dependent upon the reaction of the medium. A decidedly acid reaction favors the formation of acetone and decreases the production of acid.

TABLE VIII.

Comparison of Products of Fermentation at Different Reactions.
Calculated for 100 Cc. of Culture.

Carbohydrate.	Reaction.	Volatile acid as acetic.		Ethyl alcohol.		Acetone.		Total products.
	pH	*gm.*	*per cent**	*gm.*	*per cent**	*gm.*	*per cent**	*gm.*
Glucose............	5.5	0.2364	51.5	0.1766	38.5	0.0459	10.0	0.4589
"	6.5	0.4116	55.3	0.2493	33.4	0.0844	11.3	0.7453
"	7.5	0.4056	56.1	0.2641	36.5	0.0536	7.4	0.7233
"	8.5	0.6522	64.3	0.3220	31.7	0.0413	4.0	1.0155

* These figures represent the percentage of the total products obtained.

Fermentation of Potato Starch in the Absence of Calcium Carbonate.

The object of this experiment was to determine the rate of acid, alcohol, and acetone production. A medium of the following composition was prepared: 5 liters of city tap water, 50 gm. of peptone, 5 gm. of di-basic potassium phosphate, and 100 gm.

of potato starch. 500 cc. of this medium were removed and 100 cc. aliquots of it were transferred to 150 cc. Erlenmeyer flasks. These flasks were sterilized at 15 pounds pressure for 30 minutes and then inoculated with *Bacillus acetoethylicum* and incubated at 37°C. for 52 hours. 100 cc. of this inoculum were added to the remaining sterile 4,500 cc. of medium in a 5 liter flask and incubated at 37°C. The flask was never removed from the incubator during the time of active fermentation. A complete hydrolysis of the starch and vigorous fermentation and evolution of gas had taken place within 18 hours, at which time the first analysis was made. At various intervals from this time, 180 cc. portions of the culture were withdrawn under sterile conditions. Duplicate portions of 10 cc. each were titrated directly with 0.1 N barium hydroxide, and 50 cc. were acidified with sulfuric acid and subjected to steam distillation for the determination of volatile acids. The non-volatile acid was determined in a few cases on the residue from the above distillations as previously described. The alcohol was determined on 100 cc. of culture as outlined in the first experiment. Tests for acetone were made on portions of the alcohol distillate, but no positive tests were obtained until the time indicated on the chart. No acetone could be detected until 299 hours after inoculation. A well defined test was obtained at the end of 395 hours but is not indicated in Fig. 2. This production of acetone at these points seems to be coincident with the fall in acidity. Speakman (1920), working with *Bacillus granulobacter pectinovorum*, has observed the same phenomenon and makes the statement that without this fall of acidity no acetone is produced. He also obtained an increase in acetone production by adding various organic acids to his media, which would further substantiate the theory that acetone is produced from acetic or other acids. His experiments show that in the higher fatty acid series the yield of acetone is diminished in a regular manner, and the products of alcohol increase.

From unpublished data it has been found that acetaldehyde is formed as an intermediate product, and it is possible that the acetone is produced from this compound by condensation, oxidation, and decarboxylation. Raistrick and Clark (1919) state that it is one of Weizmann's unpublished observations that *Bacillus granulobacter pectinovorum* probably produces acetone from the carbohydrates of maize by the decarboxylation of aceto-acetic acid. The exact nature of the mechanism of acetone formation is still unsolved. The data on which Fig. 2 is constructed are given in Table IX.

The hydrolysis of potato starch in the first stages of fermentation presents another interesting problem. These hydrolytic changes, indicating enzyme activity, were observed about 12 hours after inoculation, and continued until the solution was perfectly clear at the end of about 36 hours. Potato starch medium, treated with fresh inoculum and toluene, when incubated, showed similar changes. Starch medium, to which was added a portion of an active culture, which had been passed through a Berkefeld filter, also showed enzyme activity.

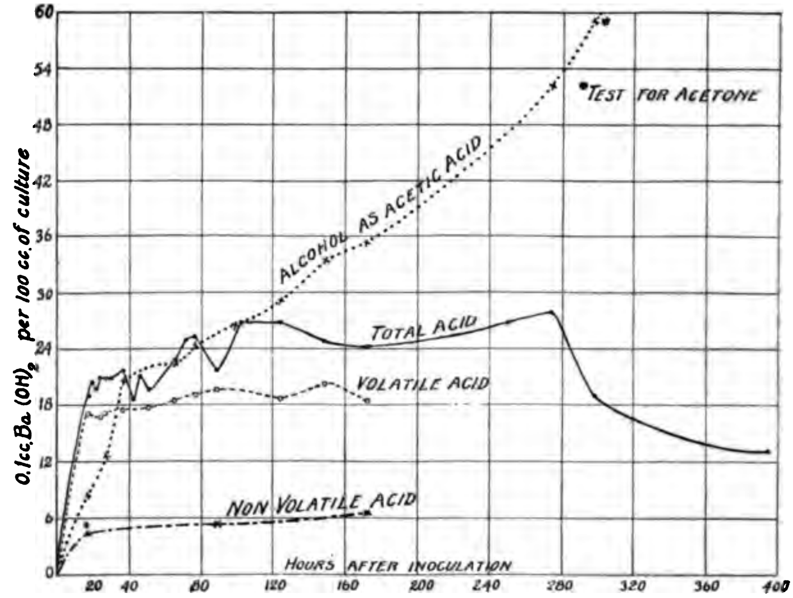


FIG. 2. Curves showing the rate at which different products are formed.

Samples of the above cultures produced a red color with iodine solution, and a finely divided suspension with two volumes of acetone or alcohol, showing the presence of some dextrins.

These experiments, though not conclusive, indicate that some enzymes are secreted which liquefy and hydrolyze the starch probably into dextrin and sugars, which are then utilized by the organism in the formation of the various products of fermentation.

Samples of a fermenting potato starch culture, when tested with Fehling's solution, showed no sugar. Determinations on aliquots of solutions of the above experiments yielded only traces of a reducing sugar. If reducing sugars are formed in the fermenting culture they are fermented by the organism so rapidly that none accumulates in the culture.

TABLE IX.

Rate of Formation of Different Products.

Date.	Time after inoculation.	0.1 N acid in 100 cc. of culture.			
		Total.	Volatile acid.	Non-volatile acid.	Volatile acid from alcohol.
1920	*hrs.*	*cc.*	*cc.*	*cc.*	*cc.*
Apr. 14, 9 a.m.	18	19.2	17.2	4.44	8.6
12 m.	20	20.6			
2 p.m.	22	19.7			
4 "	24	21.0	16.8		12.6
8 "	28	21.0	17.0		
10 "	30	21.0			
Apr. 15, 6 a.m.	38	21.6	17.4		20.6
10 "	42	18.8			
2 p.m.	46	21.3			
7 "	51	19.7	17.8		
Apr. 16, 9 a.m.	65	23.2	18.6		22.4
4 p.m.	72	25.0			
9 "	77	25.3	19.4		24.4
Apr. 17, 9 a.m.	89	22.1	19.8	5.42	26.6
10 p.m.	102	27.0			
Apr. 18, 9 "	125	27.1	18.8		29.1
" 19, 9 "	149	25.0	20.4		33.8
" 20, 9 "	173	24.3	18.2	6.58	35.4
" 22, 9 "	251	27.1			
" 23, 9 "	275	28.1			52.6
" 24, 9 "	299	19.1			59.0
" 28, 9 a.m.	395	13.1			

CONCLUSIONS.

Bacillus acetoethylicum ferments glucose, sucrose, potato starch, and xylose in a peptone-phosphate medium with the production of acetic, formic, and lactic acids, ethyl alcohol, acetone, and carbon dioxide. These products represent from 90 to 95 per cent of the sugar fermented. In a 2 per cent solution 75 to 80 per cent of the original carbohydrate is fermented within 10 to 15 days.

The optimum condition for acetone production is at a pH of 5.8 to 6.0. The influence of the reaction of the medium has a very marked effect upon the products formed. An alkaline medium maintained at about pH 8.0 is fermented by *Bacillus acetoethylicum* with the formation of large amounts of volatile acids, and comparatively small amounts of alcohol and acetone. A balance must therefore exist between these three products in the fermentation of the above carbohydrates. An increase in the volatile acid content signifies a corresponding decrease in the amount of alcohol and acetone present. *Vice versa*, an increase of acetone and alcohol, where the fermentation takes place under optimum conditions for the production of these products, is accompanied by a decrease in the yield of volatile acids. Acetone is not produced immediately in new cultures, but is formed after several days of fermentation.

The volatile acid formed is composed of about 45 per cent formic and about 55 per cent acetic.

Ethyl alcohol, with traces of some higher alcohols, is produced in amounts varying from 8 to 25 per cent, depending upon the reaction of the fermenting medium.

Small amounts of non-volatile acid are produced; this acid has been shown to be lactic.

Potato starch medium when inoculated with *Bacillus acetoethylicum* is first liquefied and then goes through a process of hydrolysis. No reducing sugars have been detected in a fermenting culture of this kind. Cultures previously treated with toluene, or freed from organisms by filtration, also produced similar changes in a starch medium. Traces of reducing sugars were found, and a characteristic red color was always produced when treated with iodine solution. The hydrolysis of starch into dextrin-like substances indicates the liberation of enzymes by the bacterial cell which function as hydrolytic agents. The negative tests for reducing sugar do not exclude the probability of their formation during bacterial activity, for the sugars may be utilized by the organism as rapidly as they are formed.

BIBLIOGRAPHY.

Brown, H. T., Morris, G. H., and Millar, J. H., *J. Chem. Soc.*, 1897, lxxi, 275.

Dox, A. W., and Lamb, A. R., *J. Am. Chem. Soc.*, 1916, xxxviii, 2561.

Fernbach, A., and Strange, E. H., British Pat. No. 21,073, 1912.

Fincke, H., *Biochem. Z.*, 1913, li, 253.

Gillespie, L. J., and Walters, E. H., *J. Am. Chem. Soc.*, 1917, xxxix, 2027.

Goodwin, L. F., *J. Am. Chem. Soc.*, 1920, xlii, 39.

Northrop, J. H., Ashe, L. H., and Senior, J. H., *J. Biol. Chem.*, 1919, xxxix, 1.

Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xli, 431.

Raistrick, H., and Clark, A. B., *Biochem. J.*, 1919, xiii, 329.

Reilly, J., Hickinbottom, W. J., Henley, F. R., and Thaysen, A. C., *Biochem. J.*, 1920, xiv, 229.

Schardinger, F., *Centr. Bakt.*, *2te Abt.*, 1905, xiv, 772.

Speakman, H. B., *J. Biol. Chem.*, 1920, xli, 319.

Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Weizmann, C., British Pat. No. 4,845, 1915; U. S. Pat. No. 1,315,585, 1919.

DIHYDROXYPHENYLALANINE, A CONSTITUENT OF THE VELVET BEAN.*

BY EMERSON R. MILLER.

(*From the Alabama Agricultural Experiment Station, Auburn.*)

(Received for publication, September 23, 1920.)

Shortly after beginning a chemical investigation of the velvet bean *Stizolobium deeringianum* Bort the writer observed that an aqueous extract of the beans soon became colored when exposed to the air, passing through several shades of color, finally becoming black.

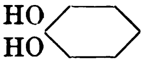
It was thought at first that this behavior was due to the action of an oxidizing enzyme upon a *p*-hydroxyphenyl compound occurring in the beans, but it later became evident that an enzyme is not necessarily concerned in the reaction. This conclusion is supported by the fact that an extract of the beans prepared by boiling with strong alcohol, filtering, and evaporating to dryness will in a short time turn black if mixed with water and kept exposed to the air.

Further examination developed the interesting fact that both the aqueous and the alcoholic extracts react with ferric chloride producing a light green color which changes to a beautiful reddish purple upon the addition of sodium carbonate or other alkali or by the addition of sodium acetate.

It was also observed that both aqueous and alcoholic extracts became colored reddish to brown immediately upon addition of an alkali, the degree and shade of color depending upon the strength and concentration of the alkali. This phenolic substance is sensitive to such weak organic bases as pyridine and quinoline. The moist powdered beans when mixed with either of these bases and exposed to the air became colored more rapidly than the moist powder alone. With strong organic bases such as piperidine and benzylamine the production of color is much more pronounced.

---

* Published with the permission of the Director of the Alabama Agricultural Experiment Station.

These reactions indicate that the phenolic substance is either pyrocatechin or a pyrocatechin derivative. The fact that an alcoholic extract which gave no reaction for protein did give a pronounced test for nitrogen suggested that the compound in question might be an amino-acid and at this point it was learned that Torquati,[1] in 1913, had separated from seedlings and from the pods of *Vicia faba* a nitrogenous substance which in its chemical reactions showed striking resemblances to this phenolic substance now separated from velvet beans. Also that Guggenheim[2] a little later had separated the substance from the pods of *Vicia faba* and had identified it as the amino-acid, 3-4-dihydroxyphenylalanine,  $-CH_2-CH(NH_2)COOH$, which Funk[3] had synthesized in 1911, starting with 3-4-carbonyldihydroxybenzaldehyde and hippuric acid, following the method of Erlenmeyer and Halsey[4] for the synthesis of tyrosine.

Separation of the Phenolic Substance.

Following, in the main, the methods of Torquati and Guggenheim, the author extracted a quantity of the powdered beans by maceration and percolation with a weak aqueous solution of sulfur dioxide containing a small amount of acetic acid. The clear liquid which resulted was then treated with a 20 per cent solution of neutral lead acetate as long as a precipitate was formed. After filtration, which in some cases was difficult, the filtrate was made slightly alkaline with ammonia water, which caused precipitation of the lead compound of the amino-acid. This was filtered off, washed, suspended in water, and decomposed by a current of hydrogen sulfide. After removal of the precipitated lead sulfide, the filtrate was evaporated to dryness on a water bath under diminished pressure in a current of carbon dioxide. The residue was crystallized from hot dilute alcohol. The crystalline substance was found to have a melting point of 275–276° with decomposition. The compound obtained by Torquati melted at 273–275° and Guggenheim reported the melting point of his compound to be 280°.

---

[1] Torquati, T., *Arch. farm. sperim.*, 1913, xv, 213, 308.

[2] Guggenheim, M., *Z. physiol. Chem.*, 1913, lxxxviii, 276.

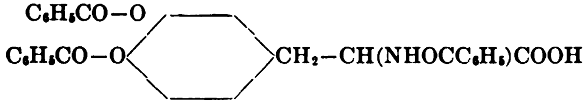
[3] Funk, C., *J. Chem. Soc.*, 1911, xcix, 554.

[4] Erlenmeyer, E., Jr., and Halsey, J. T., *Ann. Chem.*, 1899, cccvii, 138; *Ber. chem. Ges.*, 1897, xxx, 2981.

Preparation of the Tribenzoyl Derivative.

1.5 gm. of the amino-acid in 300 cc. of a 10 per cent aqueous solution of sodium bicarbonate were mixed with 9.5 gm. of benzoyl chloride and shaken until the odor of the latter had practically disappeared. The brown, amorphous mass which separated was filtered off, washed, dissolved in glacial acetic acid, and again precipitated by pouring the solution into water. The substance which separated was filtered off and crystallized from alcohol. After recrystallization the substance was obtained in the form of small colorless needles. The melting point was found to be fairly sharp at 170°, with some decomposition. For the corresponding derivative of his compound Guggenheim reported the melting point 170° (not sharp).

A Kjeldahl nitrogen estimation of the tribenzoyl derivative gave the following result. 0.287 gm. of the substance yielded sufficient ammonia to neutralize 6.0 cc. of 0.1 N sulfuric acid, corresponding to 2.92 per cent of nitrogen. Guggenheim found 2.9 per cent of nitrogen in his tribenzoyl derivative. The formula requires 2.75 per cent of nitrogen. In the present case not all the substance used for the nitrogen determination was as pure as the portion used for the melting point determination, which probably accounts for the somewhat high result.



Conversion into Protocatechuic Acid.

A small amount of the substance under investigation was fused with about ten times its own weight of potassium hydroxide, cooled, taken up in water, acidulated with sulfuric acid, and extracted with ether. After standing a short time there was formed in the thick, dark brown residue left by distilling off the ether a quantity of fine needle-shaped crystals. These were found to be readily soluble in water or alcohol, somewhat less so in ether, and very sparingly soluble in boiling benzene by means of which the substance was separated and purified. The melting point was found to be 197°. For his compound Guggenheim found the melting point 195°. Works of reference generally give the melting point of protocatechuic acid as 198–199°.

The substance obtained by this alkali fusion also agreed with protocatechuic acid in that it gave with ferric chloride a bluish green color which was changed to a deep red upon the addition of sodium carbonate.

Solubility and Reactions of the Amino-Acid.

So far water seems to be the best solvent for this compound since no other solvent used extracts it from the powdered beans so readily; however, on account of its instability in water in the presence of air an effort was made to find an organic solvent for it. Of a great many liquids tried only ethyl and methyl alcohols could be used. In ether, petroleum ether, acetic ether, chloroform, benzene, acetone, and many others it is practically insoluble. With dry powdered beans and dry solvent it is not extracted even by boiling with the liquids named.

Both methyl and ethyl alcohol extract it in the cold, though very slowly. The process is hastened by the use of heat, but even at the boiling point these solvents extract it slowly. Extraction with hot 50 to 75 per cent alcohol will probably prove to be most satisfactory.

The identity of the compound separated from velvet beans with that separated by Torquati and Guggenheim from the pods of *Vicia faba* is further shown by complete agreement of their chemical reactions, as follows:

1. With ferric chloride it produces a green color which is changed to reddish purple upon the addition of an alkali.
2. With alkalies alone it turns reddish brown, finally black.
3. It immediately reduces an ammoniacal solution of silver nitrate.

4. An aqueous solution of the amino-acid dissolves freshly prepared copper carbonate forming a blue or greenish blue solution in which a black flocculent substance quickly forms.

5. An aqueous solution slightly acidulated with sulfuric acid immediately decolorizes a solution of potassium permanganate.

6. With a solution of mercuric chloride in distilled water it seems to produce no color, but upon the addition of a small amount of sodium acetate there is produced a bright red color. Usually if hydrant water is used the red color is produced at once.

7. When a small amount of the substance in aqueous solution is added to a solution of potassium ferricyanide containing some ferric chloride a blue or greenish blue color is produced immediately, showing that reduction has occurred.

8. With a dilute solution of iodine, or bromine or iodic acid, it produces a pink to red solution depending upon concentration.

9. With potassium ferricyanide solution it produces a deep red color.

It is of some interest to note that in all these reactions the amino-acid behaves like the base adrenalin.

10. An aqueous solution of this substance produces decided effervescence when mixed with nitrous acid. This is in harmony with its amino-acid character.

Presence of the Amino-Acid in Other Varieties of Velvet Beans.

So far the writer has actually separated the previously mentioned amino-acid only from the seed of the Early Speckled or Georgia velvet bean, but most of the reactions given above were applied to aqueous extracts of twenty-six varieties of velvet beans obtained from the Alabama, Georgia, and Florida Agricultural Experiment Stations. In all cases the characteristic reactions of the amino-acid were obtained. The same reactions were also obtained with extracts of the Yokohama[5] and Lyon[6] beans. It is therefore highly probable that 3-4-dihydroxyphenylalanine is a characteristic constituent of the seeds of plants of the genus *Stizolobium.*

Seeds from several species of other genera of the family Leguminosæ were tested in the same way but with negative results.

---

[5] *Stizolobium hassjoo* Piper and Tracy.

[6] *Stizolobium niveum* (Roxburgh) Kuntze.

Pharmacological Action.

Of even greater interest than the chemical constitution of this substance is the question of its pharmacological action.

As the result of experiments upon guinea pigs Guggenheim concluded that the substance is rather indifferent pharmacologically.

However, in an experiment upon himself in which he took 2.5 gm. by mouth he experienced great distress within about 10 minutes and vomited twice, thus showing that the substance is not wholly harmless.

At the Florida Agricultural Experiment Station several persons ate small amounts of cooked Lyon beans with the result that some who ate of them experienced both vomiting and purging.

When one considers that this substance is rather closely related chemically to the powerful base adrenalin it is quite probable that long continued feeding of velvet beans might cause such harmful results as have been observed.

Further work upon this amino-acid is in progress.

A TEST FOR ANTIBERI-BERI VITAMINE AND ITS PRACTICAL APPLICATION.*

By CASIMIR FUNK and HARRY E. DUBIN.

(*From the Research Laboratory of H. A. Metz, New York.*)

PLATE 1.

(Received for publication, August 11, 1920.)

INTRODUCTION.

In 1912, one of us (C. F.) experimented with antiberi-beri vitamine to see if it could act as a coferment in alcoholic fermentation. The experiments were unsuccessful at that time, but now through the work of Williams,[1] Bachmann,[2] and Eddy and Stevenson[3] the subject has attained new interest. Their results seem to prove that vitamine activity can be measured by the growth of yeast cells, and would permit of dispensing with animal experimentation in the initial stages of vitamine fractionation.

Because of the uncertainty of the Bachmann fermentation test and the complicated procedure of Eddy's test, it was thought desirable to develop a more simple method which would facilitate our contemplated vitamine fractionation work, which we shall discuss in detail later on.

Since our preliminary communication, Williams[4] has published a method somewhat analogous to ours but not quite so easily or so quickly manipulated.

---

* Read before the Society for Experimental Biology and Medicine, New Haven, May 22, 1920.

[1] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

[2] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

[3] Eddy, W. H., and Stevenson, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 122.

[4] Williams, R. J., *J. Biol. Chem.*, 1920, xlii, 259.

Method.

Our procedure is as follows. A yeast suspension is prepared by shaking a loopful of a 48 hours pure yeast culture in 100 cc. of Nägeli solution on a shaking machine for 3 hours. Duplicate sets of tubes (ordinary bacteriological test-tubes) are prepared containing:

(1) 4 cc. yeast suspension + 5 cc. Nägeli + 1 cc. water.

(2) 1 cc. unknown vitamine solution + 5 cc. Nägeli + 4 cc. water.

(3) 1 cc. unknown vitamine solution + 5 cc. Nägeli + 4 cc. yeast suspension.

The tubes are incubated for 20 hours at 30°C., and the fermentation is then interrupted by heating the contents of the tubes to 75°C. in a water bath for a few minutes. This step is of importance as otherwise the continuing fermentation would interfere with the subsequent centrifugation. The contents of the tubes are now transferred to special centrifuge tubes, the bottom part of which ends in a capillary 2.5 cm. long and divided into mm., as shown in the full size photograph (Fig. 1). The transfer is made quantitatively by washing four times with 1 cc. portions of water. A piece of wire about half the size of the capillary is then used to force the air out of the capillary of the centrifuge tube, and the wire washed with a few drops of water.

The tubes are now centrifuged at about 2,500 revolutions per minute for 15 minutes, and the reading on the capillary is made without much delay as there is a tendency for the yeast cells to swell slightly after a time. The centrifuge tubes are easily cleaned with the aid of the piece of wire, used as a ramrod.

The reading of Tube 1 constitutes the blank and must be subtracted from the reading of Tube 3; Tube 2 shows no growth of yeast cells and is included in the test merely as an extra control. Sterility during the entire process, excepting centrifugation, is of paramount importance. A microscopic examination of the centrifuged mass showed well formed yeast cells only.

The above procedure was so well standardized that the controls with yeast suspension and Nägeli alone were practically constant at 3.5 mm. Tests which did not give a reading of at least 4.5 mm. were considered to indicate the inactivity of the substance the vitamine content of which was being investigated. Duplicate experiments checked very well, so that the method appeared to be applicable for a quantitative determination. It was noticed, however, that the amount of growth of yeast cells obtained, as shown by the reading of the centrifuge tube, does not double if the amount of vitamine is doubled.

By trying varying amounts of vitamine, a curve was established—using autolyzed yeast as a source of vitamine and as a standard—by which it is possible to approximate the vitamine content of an unknown substance in terms of the standard, autolyzed yeast.

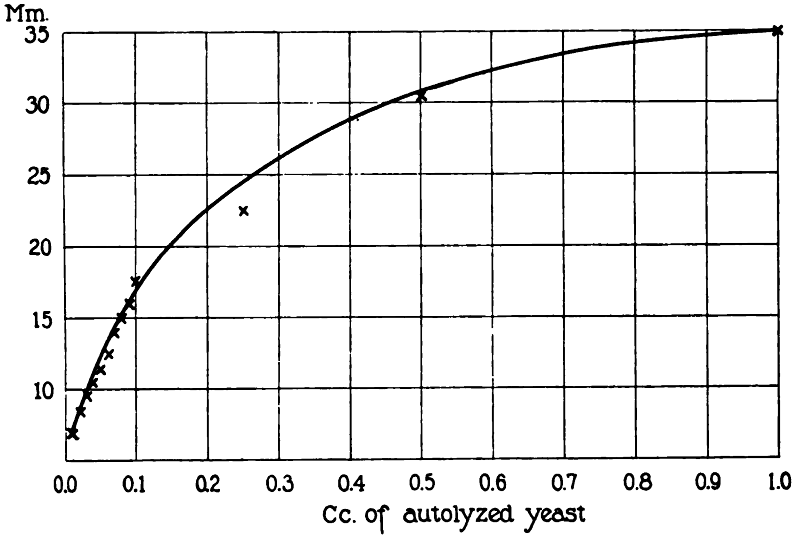


Chart 1. Vitamine activity shown by increasing amounts of autolyzed yeast.

The method is sensitive to 0.0001 cc. of autolyzed yeast, but the test is best arranged so that the amount of unknown substance used corresponds to 0.05 cc. of autolyzed yeast, since in the interval between 0.01 and 0.1 cc. of autolyzed yeast the curve climbs up abruptly and small differences can be read with facility.

It will be noticed (Chart 1) that although the curve as a whole tends to flatten out, that part of the curve between 0.01 and 0.1 cc. is practically a straight line, and it is this part of the curve, plotted separately (Chart 2), that is best used in determining the activity of a substance. Should the test yield a too voluminous growth of yeast cells, the solution being tested may be diluted so that the amount taken for a test (1 cc.) will give a result that will be readable on Chart 2.

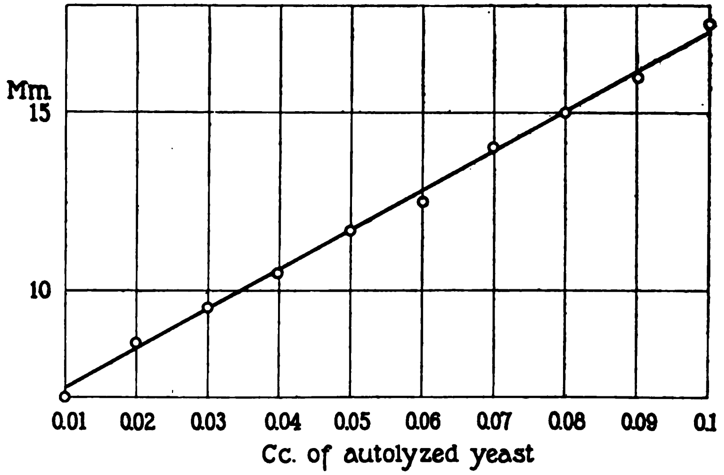


CHART 2. Vitamine activity shown by increasing amounts of autolyzed yeast.

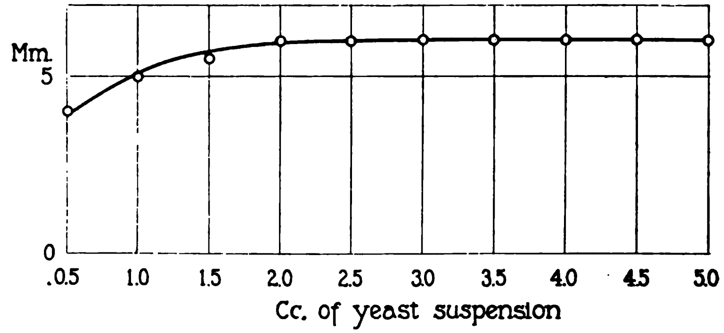


CHART 3. Effect of varying amounts of yeast suspension.

In order to determine whether or not the amount of yeast suspension used in the test has any effect upon the final results, a series of experiments was made in which the amount of autolyzed yeast was constant (0.01 cc.), while the quantity of yeast suspension was varied from 0.5 to 5.0 cc. Chart 3 shows that the variation of the amount of yeast suspension has very little effect on the final result, as obtained by our method, whereas in Eddy's method the number of cells incubated varies the controls to an undesirable extent.

DISCUSSION.

Using, as we did, autolyzed yeast as a source of antiberi-beri vitamine, we have evidence in the flattening out of our curve that there are inhibiting substances present. In no case did we find an exact direct proportionality. This shows that at present the method cannot be applied for the purpose of comparing the vitamine content of different substances, because not only the amount of vitamine but also the inhibitory substances may vary. It may be noted here that Williams[4] did obtain a proportionality in certain concentrations, using a vitamine solution of unstated origin, but he also notices the presence of inhibitory substances. At any rate, for the purposes of approximating the vitamine activity under usual conditions, our yeast curve is of greater advantage than that of Williams because the natural yeast extract used compares with natural extracts of substances to be tested.

We must, of course, be careful in interpreting results obtained by any of the present methods. A negative result may simply mean the presence of inhibitory substances, whereas a positive result very likely indicates the presence of antiberi-beri or B vitamine. However, in working with one substance only, as for example autolyzed yeast, the results obtained are of real value in showing the extent of activity and the removal of inhibitory substances at various stages of yeast fractionation and purification.

Our findings suggest that in curing beri-beri pigeons or in studying growth in rats, we may encounter the same difficulty; namely, that treating with crude vitamine-containing substances may give us the results as affected by the presence of inhibiting elements, and may not be at all indicative of the true value of the substance fed.

We feel also that a quantitative estimation of the vitamine content of any product is possible only if the factor of inhibition can be removed or obviated.

Specificity Tests.

After a method well suited to our needs had been developed, it was then believed worth while to determine how far the test is specific for antiberi-beri vitamine. To this end, various substances were tried with results as shown in Table I.

Very small amounts of purified vitamine fractions from yeast prepared by Funk[5] in 1912 and analyzed in 1913 still retained their activity though somewhat attenuated. Eddy has likewise tested these fractions with similar results.

Urine exhibited activity, thus corroborating Muckenfuss[6] and Gaglio,[7] who also found vitamine in urine. This test may prove to be of clinical value later on, but possibly blood could be used to greater advantage for diagnostic purposes.

Saliva was found inactive, and this was also the case with polished rice, in spite of the recent statement of Green[8] to the contrary.

Corn and wheat, separated from the germ by the method of Voegtlin and Myers[9] still exhibited considerable activity although about ten times less than that shown by the germ-containing portion.

Among the substances showing no activity were leucine, tryptophane, allantoin, hydantoin, nicotinic acid, a mixture of ten purine and pyrimidine bases, and a mixture of six amino-acids.

Extracted meat and starch, Crisco and lard, autoclaved at 30 pounds for 3 hours, showed no activity. These extracted and autoclaved foodstuffs are now being used in our laboratory for dietary experiments with rats. We are using this method of establishing the absence of vitamine B from the experimental diets.

Orange juice was found to be active but on shaking with fullers' earth (50 gm. per liter) for 6 hours it lost some of its activity; shaking with 200 gm. per liter removed all the active substance from orange juice.

---

[5] Funk, C., *J. Physiol.*, 1912–13, xlv, 75; 1913, xlvi, 173.

[6] Muckenfuss, A. M., *J. Am. Chem. Soc.*, 1918, xl, 1606.

[7] Gaglio, G., *Policlinico*, 1919, xxvi, 1381.

[8] Green, H. H., *South African J. Sc.*, 1918, xiv, 483.

[9] Voegtlin, C., and Myers, C. N., *Am. J. Physiol.*, 1919, xlviii, 504.

TABLE I.

Specificity Tests.

No.	Substance tested.	Activity.*
		mm.
1	0.6 mg. crude vitamine fraction (1912)	2.0
2	0.4 “ “ “ “ (1913, No. 1)	1.0
3	0.4 “ “ “ “ (1913, No. 2)	2.5
4	1 cc. urine	4.0
5	12 mg. thyroid (desiccated)	2.0
6	75 cc. pituitary	2.0
7	100 mg. corn (minus the germ)	5.0
8	Corn germ	10.0
9	100 mg. wheat (minus the germ)	3.5
10	100 “ “ germ	6.5
11	1 cc. orange juice	10.5
12	1 “ “ “ shaken for 6 hrs. with fullers' earth (50 gm. per liter)	3.5
13	1 cc. orange juice shaken for 6 hrs. with fullers' earth (200 gm. per liter)	0†
14	0.05 cc. autolyzed yeast (protein-free)	11.5
15	0.05 “ “ “ shaken with fullers' earth (50 gm. per liter) for 6 hrs	6.0
16	0.05 cc. autolyzed yeast shaken with fullers' earth (200 gm. per liter) for 6 hrs	3.5
17	150 mg. polished rice	0
18	20 “ hydantoin	Negative.‡
19	20 “ nicotinic acid	0
20	20 “ leucine	0
21	20 “ allantoin	0
22	1 cc. saliva	Negative.
23	3 mg. pilocarpine	0
24	10 “ tryptophane	0
25	0.01 cc. cod liver oil (watery extract)	0
26	1 cc. of a solution representing 10 mg. each of a mixture of ten purine and pyrimidine bases	Negative.
27	1 cc. of a solution representing 10 mg. each of a mixture of six amino-acids	0

* It should be borne in mind that a blank determination is run with every test and that the figures in the "activity" column represent actual readings with the blank already subtracted.

† A "zero" reading means that the actual reading was exactly the same as the blank.

‡ A "negative" result signifies that the actual reading obtained was less than the blank, which would indicate that there has been some inhibition which retarded the growth of the yeast cells.

Autolyzed yeast, shaken with fullers' earth (200 gm. per liter), had lost the greater part of its activity; larger amounts of fullers' earth would undoubtedly remove all the antiberi-beri vitamine.

A watery extract of cod liver oil proved to be inactive.

Pilocarpine, found by Dutcher[10] to be curative for beri-beri pigeons, showed no activity, while desiccated thyroid gland was active, as it contained some cellular constituents. Pituitary gland was likewise found active, for the same reason. These alleged curing experiments with pilocarpine and thyroxin are most likely due to stimulation of metabolism and setting free of the vitamine in the organs.

Influence of Various Reagents and Procedures on the Vitamine of Autolyzed Yeast.

As a preliminary to a systematic fractionation of autolyzed yeast, we studied the activity of the yeast under varying conditions. A definite amount of the yeast was treated in a certain way after which its activity was tested, and, since the quantity of yeast ultimately taken for a test always corresponded to 0.05 cc. of the original yeast, the results obtained were directly comparable with each other. The following is a brief résumé of our findings, as recorded in Table II.

1. Oxidation with oxygen and platinum sponge has no effect upon the activity.
2. Reduction with palladium chloride and hydrogen increases the activity, either because of a true reduction of the vitamine or because of the removal of some inhibiting substances.
3. Oxidation with potassium permanganate in neutral and in acid media reduces the activity.
4. Oxidation with hydrogen peroxide prevents the growth of yeast cells, but if the peroxide is removed before making the test then no lessening in the activity results. This indicates that the mere presence of peroxide, and likely of permanganate too, serves to prevent the growth of yeast cells.
5. Reduction with zinc dust and sulfuric acid increases the activity.

---

[10] Dutcher, R. A., *J. Biol. Chem.*, 1919, xxxix, 63.

6. Addition of zinc dust to yeast has no effect upon the activity.

7. After precipitating with lead acetate, removing the lead with H_2S, there is a marked increase of activity due undoubtedly to the elimination of certain impurities.

8. Precipitation with silver acetate slightly reduces the activity.

9. Both mercuric sulfate and mercuric chloride precipitations increase the activity.

TABLE II.

*Influence of Various Reagents and Procedures on the Activity of Autolyzed Yeast.**

No.	Method of treatment.	Activity.
		mm.
1	Filtered through Berkefeld filter	10.5
2	Protein removed	10.5
3	Oxidation with platinum sponge and oxygen for 8 hrs.	10.5
4	Reduction with palladium chloride and hydrogen for 8 hrs	14.0
5	Treatment with nitrous acid	11.5
6	Oxidation with hydrogen peroxide	Negative.
7	Same as No. 6 but with peroxide removed before testing	10.5
8	Reduction with zinc dust and sulfuric acid	15.0
9	Treatment " " " alone	10.5
10	Boiling for 3 hrs. with acetic anhydride	Negative.
11	Oxidation with potassium permanganate	1.5
12	Same as No. 11 in the presence of sulfuric acid	3.0
13	Lead acetate	20.0
14	Silver "	8.0
15	Mercuric sulfate	9.5
16	" chloride	12.0
17	Extracted with ether and ether extract tested	1.0

* See foot-notes to Tables I and III.

10. Nitrous acid increases the activity somewhat. McCollum and Simmonds[11] also have found that B vitamine is not affected by nitrous acid.

11. Ether of itself extracts nothing that is active from autolyzed yeast, but to the slight extent that yeast is soluble in ether, the ether extract shows a correspondingly slight activity.

---

[11] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxiii, 55.

Fractionation of Autolyzed Yeast.

The starting point in this work was an extract of autolyzed yeast, because such an extract is very rich in antiberi-beri vitamine. For example, two extracts were prepared; (1) by the method of Osborne and Wakeman,[12] and (2) by our method which consisted of autolyzing the yeast and then boiling it to remove the coagulable protein. The same quantity of yeast was used in each case and the final concentration in each was the same. It is of interest, therefore, to note that whereas the first extract—according to the method of Osborne and Wakeman—contained only one-fifth of the nitrogenous substances present in the second extract, the latter was many times more active than the former in spite of the large amount of impurities present. It was this finding which prompted us to autolyze the yeast before extracting it.

The subsequent fractionation was carried out as follows:

Starting with a definite amount of yeast extract the nitrogen content of which had been determined and the vitamine activity of which had been established by the method just described, lead acetate was added to complete precipitation, and the excess lead removed with H_2S, which was in turn removed by vacuum evaporation. The solution was then made up to original volume and the nitrogen content and vitamine activity were again determined. The same procedure was then repeated with mercuric sulfate, silver acetate, and mercuric chloride, respectively, the nitrogen content and vitamine activity being determined each time, after eliminating the precipitating reagent and making up to the original volume.

The solution was then acidified with HCl and treated with nitrous oxide, after which it was extracted with ether, and the ether washed with water, the washings being added to the extracted solution. The nitrogen and vitamine activity were again determined. The vitamine activity of the ether extract was also determined.

The final step was the reduction of the solution with hydrogen and palladium. The results obtained are summarized in Table III from which it may be seen that the total nitrogen content has been reduced from 14.89 to 4.55 gm., while the vitamine activity has been reduced from a reading of 10.5 to 5.5 mm. When it is considered that the original yeast had been manipulated for a period of 12 weeks through a series of four precipitations, a diazotization, and a reduction, together with unavoidable small losses at every stage, it is indeed worthy of record that the loss of activity was no greater than that found. As for the loss in activity during the diazotization, we have learned the cause and eliminated it in our subsequent work so that our end-product

---

[12] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl. 383.

TABLE III.

Activity of Autolyzed Yeast at Various Stages of Purification.

No.	Substance tested and procedure employed.*	Nitrogen.	Activity.
		gm	*mm.*
1	Original protein-free yeast	14.89	10.5
2	No. 1 plus lead acetate—filtrate	12.84	20.0
3	Concentration of filtrate of No. 2 yielded 6 gm. of substance which crystallized out—10 mg		Negative.
4	No. 2 filtrate plus mercuric sulfate—filtrate	12.18	9.5
5	Mercuric sulfate precipitate decomposed and dissolved in water		2.5
6	No. 4 filtrate plus silver acetate—filtrate	11.20	8.0
7	Silver acetate precipitate decomposed and dissolved in water		3.5
8	No. 6 filtrate plus mercuric chloride—filtrate	10.3	12.0
9	Mercuric chloride precipitate decomposed and dissolved in water		Negative.
10	No. 8 filtrate plus nitrous acid	4.55	4.0†
11	Ether extract of No. 10		Negative.
12	After extracting No. 10 with ether, it was reduced with hydrogen and palladium		5.5

* All the tests made corresponded to 0.05 cc. of original protein-free autolyzed yeast, so that the activity as shown by one test is directly comparable to that of any other test.

† The reduced activity noted here is due to certain causes which will be explained in a later paper. It may be seen from No. 5, Table II, that nitrous acid materially increases the activity of the yeast.

will show even greater activity than that recorded. Incidentally the many manipulations speak well for the stability of the antiberi-beri vitamine under various conditions.

SUMMARY AND CONCLUSION.

We have described a method for testing the antiberi-beri vitamine content of various substances, using autolyzed yeast as a standard. We have used the method in determining the approximate vitamine activity of a number of products, and in observing the activity of autolyzed yeast under various conditions. By this method we have also been able to carry out a systematic fractionation of autolyzed yeast testing the vitamine activity at each stage of the purification.

Despite the apparent uncertainty surrounding the whole problem of the vitamines, it is nevertheless readily admitted that only with some such method as we have described for the testing of vitamine activity, is it possible to make greater strides in the attempt to isolate and identify the B vitamine.

Some investigators believe that the B vitamine is not identical with the substance promoting the growth of yeast. Regarding this contention, we are not prepared as yet to make any definite statement. However, the question, whether we are dealing with one vitamine—at least as found in original extracts—or with two vitamines existing side by side, is at present under further investigation, and will be reported at a later date.

EXPLANATION OF PLATE 1.

FIG. 1. The tube at the left shows the reading of the blank, while the other two tubes show the readings obtained with different amounts of autolyzed yeast.

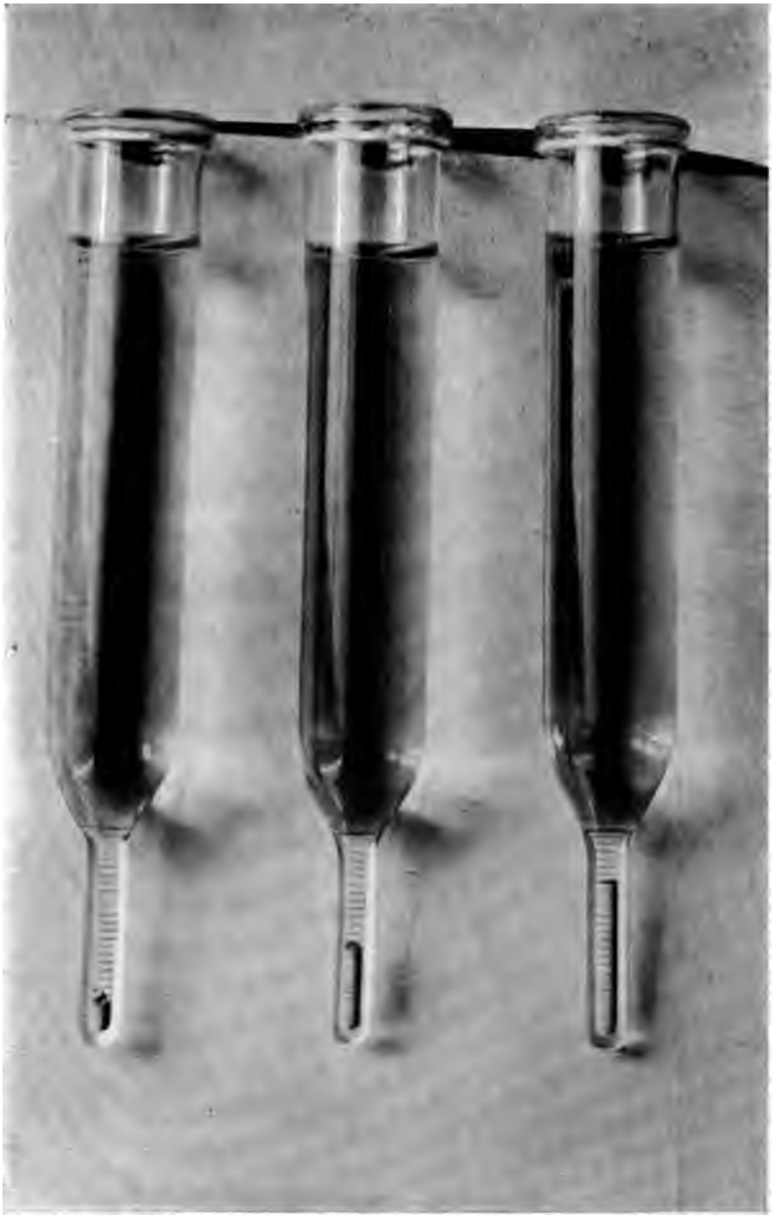


FIG. 1.

(Funk and Dubin: Antiberi-beri vitamine.)

AMINO-ACIDS OF GELATIN.

By H. D. DAKIN.

(*From Scarborough-on-Hudson.*)

(Received for publication, August 25, 1920.)

The accurate separation and estimation of the products formed by the hydrolysis of proteins still present a problem which is far from solved though many improvements have been made in recent years. The importance of the problem requires no emphasis.

In recent communications (1918, 1919) the writer has shown that certain approximate separations of amino-acids could be effected by the use of partially miscible solvents such as butyl alcohol. Under appropriate conditions the feebly ionized mono-amino-acids and proline are readily extracted while the strongly ionized bases and dicarboxylic acids remain behind. The method held out promise of material improvements in the analysis of the products of hydrolysis of proteins. With the experience gained in the use of the method in separating some of the products from casein, glutenin, and gliadin as a guide, it was decided to select a protein of medium complexity and to endeavor to separate its products of hydrolysis as completely as possible. Gelatin was the protein selected partly because existing analyses were exceptionally incomplete and also because the reputed absence of tryptophane and tyrosine appeared to simplify the problem somewhat. As a matter of fact traces of tyrosine were always found and the complications due to the presence of large amounts of glycine and hydroxyproline more than counterbalanced any advantage from the absence of tryptophane. However, a fair separation of most of the amino-acids from gelatin has been made, making up a total of somewhat over 90 per cent of the gelatin taken. Some indication has been obtained as to the nature of the remainder, while the definite absence of several amino-acids has been established whose occurrence was previously a matter of doubt.

In the following sections an account is first given of the general line of attack followed in the endeavor to separate and estimate the various amino-acids from gelatin. The separate amino-acids are then considered in detail together with an account of the practical details used in their isolation. After the individual amino-acids have been considered, a short discussion of the results is given together with a comparison of those previously recorded. Finally a description is given of the preparation and properties of a new tricyclic peptide isolated from gelatin, hydroxyprolylproline anhydride, and also the preparation of inactive proline hydantoin.

EXPERIMENTAL.

The presentation of the experimental results in a concise form has certain difficulties since they relate to something over a dozen separate analyses in each of which some particular group of amino-acids was specially investigated. The preparation of the hydrolyzed material was substantially the same in all cases and was as follows:

About 250 gm. of moist gelatin (gold or silver label) were boiled for 12 to 20 hours with sulfuric acid (300 gm.) and water (650 cc.). The solution was then transferred to a porcelain beaker and heated for 8 hours in an autoclave at 135–140°. The liquid was then diluted with hot water and barium hydroxide added until Congo paper was no longer turned blue. The barium sulfate was removed by filtration and the precipitate digested three times with additional hot water. The combined filtrates were concentrated under low pressure to a thin syrup and made up to a known volume; *e.g.*, 500 cc. The actual content of gelatin products was determined by analyzing an aliquot part for total nitrogen, adopting 18 per cent as the nitrogen content of gelatin. The solution was still fairly strongly acid to litmus and no loss of ammonia had occurred. For extraction with butyl alcohol an amount of solution equivalent to 100 to 150 gm. of gelatin was generally used. In order to avoid unnecessary repetition it may be stated at this point that evaporations of the amino-acid solutions at every stage were carried out under reduced pressure and that highly purified reagents were used for the constantly recurring precipitations. Omission of these obvious precautions lead to errors of considerable magnitude. Innumerable analyses of the various fractions were carried out by elementary combustion, by the Kjeldahl and Van Slyke methods, and by polarimetric observations. Only a few of these results are worth recording but collectively they furnished valuable information as to the progress of the various separations.

Extraction with Butyl Alcohol at Normal Pressure.

The extraction of hydrolyzed gelatin with boiling butyl alcohol under atmospheric pressure was carried out under the same conditions as those previously described in connection with casein. A Kutscher-Steudel apparatus was used and the liquid to be extracted was kept at about 60°C. by means of a bath or minute flame. A Pyrex or Jena flask was used as container for the butyl alcohol and in order to promote rapid boiling the flask was placed in a tin with high walls. The bottom of the tin was cut out with the exception of a small rim and replaced by a single layer each of iron gauze and thin asbestos paper. A fairly large Bunsen flame surrounded by a gauze jacket secured a rapid circulation of the butyl alcohol. Soon after commencing the experiment large amounts of proline were rapidly extracted and remained in solution in the butyl alcohol, while a fine granular precipitate of amino-acids separated out precisely as in the experiments with hydrolyzed casein. At intervals of 12 to 24 hours the extraction vessel was changed and the butyl alcohol renewed.

Solid Extracted Amino-Acids.—The granular precipitate of amino-acids first accumulating in the extraction flask was filtered off and washed with alcohol and dried. It was found to contain the leucine, phenylalanine, alanine, and some of the hydroxyproline. After about 48 hours extraction the character of the deposit changed and became progressively less granular, finally assuming a pasty quality. Furthermore it was found that, unlike the experiments with casein products, it was almost impossible to bring the extraction to an end within a reasonable time, so that even after a week's extraction solid amino-acids were still accumulating. The reason for this divergent behavior was found to arise from the presence of large amounts of glycine and hydroxyproline. Glycine, unlike alanine and its higher homologues, is extracted by butyl alcohol under the present conditions with great slowness, so that even after a week's extraction only about a fifth of the total glycine is removed. The hydroxyproline is more readily extracted than glycine and its extraction may be carried to virtual completion but its large amount in gelatin leads to its appearance in early and late fractions.

The following data indicate the general course of a typical extraction so far as the solid amino-acids are concerned. 115 gm. of hydrolyzed gelatin gave on extraction 36 gm. of solid amino-acids washed with alcohol and ether and dried at 110°. They contained 13.72 per cent total nitrogen and 10.52 per cent amino nitrogen the difference of 3.2 per cent being due, apparently exclusively, to hydroxyproline. The specific rotation of the mixture in 5 per cent aqueous solution was $-18.8°$. About 10 per cent of glycine (3.6 gm.) was separable as ethyl ester hydrochloride. The remainder was made up of the amino-acids already enumerated. The most painstaking examination of the aqueous liquid remaining after extraction failed to show the presence of even traces of alanine, valine, leucines, or phenylalanine, when worked up by the ester method. It therefore appeared that the solid amino-acids extracted as above described could safely be used for the estimation of these amino-acids while the estimation of glycine and hydroxyproline required other methods.

Fraction Soluble in Alcohols.—As in the experiments with hydrolyzed casein already referred to, it was found that proline from gelatin was readily extracted by butyl alcohol and naturally remained in solution. But as will be shown shortly, the material thus extracted when working under ordinary pressure is not well suited for the separation and isolation of proline.

The butyl alcoholic solutions were evaporated under reduced pressure and the dry residue was taken up in ethyl alcohol and allowed to stand for several days at 0° in order to separate traces of other acids. This separation seldom amounted to over 1 gm. per 100 gm. of gelatin and it was filtered off and added to the main bulk of solid amino-acids. The alcoholic filtrate was evaporated, taken up in water, clarified with charcoal, and filtered.

It was found that this crude "proline fraction" was incapable of much purification by using the alcohol-soluble copper salts or by using phosphotungstic acid. But a considerable purification was effected by precipitating the mixture in 10 per cent solution with saturated mercuric acetate solution (2 mols) and barium hydroxide (2 mols). The latter reagent was most conveniently dissolved in five parts of hot water, filtered, cooled with shaking, and the magma of fine crystals added with vigorous stirring to the proline and mercuric acetate solution. A bulky white precipitate containing various amino-acid impurities was produced, filtered off at once, and washed with cold water. This operation should be done quickly so as to avoid oxidation of proline by the alkaline mercuric salts. An amount of sulfuric acid roughly equivalent to the barium hydroxide was at once added and the turbid solution saturated with hydrogen sulfide in a closed flask. After filtration the liquid was exactly freed from barium or sulfuric acid and then concentrated under reduced pressure. The whole of the proline present is found in the aqueous solution, for, as Sörensen has shown, it is not precipitated by alkaline mercuric acetate like most amino-acids.

On drying the evaporated solution, a brittle hygroscopic partly crystalline mass is obtained. (The crystals were subsequently found to be largely γ-hydroxyprolylproline anhydride. See later.)

Three separate specimens on analysis gave 52.5, 52.9, 53.1 per cent carbon, 7.52, 7.67, 7.68 per cent hydrogen. The total nitrogen varied from 12.5 to 13.1, and the amino nitrogen was 0.1 to 0.2 per cent. Although the figures are fairly close to those required for proline (52.2 per cent carbon, 7.83 per cent hydrogen, 12.2 per cent nitrogen) a large part of the product was made up of cyclic anhydrides containing at least one proline nucleus. The specific rotation varied from $-52°$ to $-75°$. That this low result was not due to partial racemization of the proline was proved by the complete solubility of the copper salts in methyl alcohol. The presence of the cyclic anhydrides was proved by hydrolysis under pressure with hydrobromic acid at 140°. After successive treatments with silver oxide and hydrogen sulfide in the usual way the presence of hydroxyproline and some leucine was readily determined.

The above experiments illustrate the dangers of accepting too readily figures for the apparent proline based on purely analytical data. The amount of cyclic anhydrides (diketopiperazines) appears to increase in proportion to the length of time the butyl alcohol containing much proline is boiled with the other amino-acids in the original process of extraction under ordinary pressure. The solubility of the proline would undoubtedly favor the formation of anhydrides containing at least 1 molecule of this substance and some evidence has been obtained of the formation of prolylproline anhydride, though its complete separation has not been accomplished. In this connection Maillard's (1911) observations on the effect of heating amino-acids with glycerol at high temperatures may be recalled. As will be shown later the artificial formation of cyclic anhydrides during the extraction may be obviated by changing the conditions of extraction, although their presence due to incomplete hydrolysis of the protein has always to be reckoned with.

Amino-Acids not Extracted by Butyl Alcohol.—The aqueous solution of amino-acids remaining after extraction with butyl alcohol was examined at first in substantially the same way as that adopted with the products from casein. The bases were removed with phosphotungstic acid and then the strong acids, aspartic and glutamic acids, were largely removed by direct crystallization. Smaller additional amounts were removed by methods to be referred to later. Examination was then made for the presence of β-hydroxyglutamic acid which has been identified in casein, gliadin, and glutenin. It was definitely established that no dicarboxylic acid other than aspartic and glutamic acids was present. It is of interest to note that the large amount of amino-acids left in the mother liquor after the separation of the acids could be entirely recovered in the form of solid products with no uncrystallizable syrups. The bulk of the product was composed of glycine which was separated successively as picrate and ethyl ester, while in addition variable amounts of hydroxyproline were encountered. It is probable that a little serine may have been present but no indication was obtained of any considerable amount of amino-acids other than those first mentioned.

The results of the experiments briefly referred to in the preceding sections, which in reality represent a summary of many separate analyses, indicated that by the use of butyl alcohol as a solvent, extracting under normal pressure, a considerable separation of the amino-acids from gelatin could be effected. The main difficulties encountered were as follows: (*a*) The proline fraction was heavily contaminated with diketopiperazines, including among others hydroxyprolylproline anhydride; (*b*) glycine was only extracted to a very limited extent; (*c*) the extraction of hydroxyproline was inconveniently slow. On the other hand the amino-acids separating out in the butyl alcohol extraction flask contained practically the whole of the alanine leucine, phenylalanine, and most of the hydroxyproline and furnished excellent material for their estimation. The bases and aspartic and glutamic acids were left behind in readily separable forms together with most of the glycine and some hydroxyproline.

Extraction with Butyl and Propyl Alcohols under Reduced Pressure.

It appeared desirable to devise if possible some modification of the preceding process that would extract the proline in a purer state and secondly to improve the extraction of the hydroxyproline. The contamination of the proline with diketopiperazines obtained by extracting with butyl alcohol under ordinary pressure is apparently due in part to the prolonged boiling of the proline solution with other extracted amino-acids and hydroxyproline in particular, for the latter acid is fairly readily dissolved in the moist butyl alcohol. It appeared, therefore, that, if extraction of the proline could be effected at a lower temperature, this change could be limited. Use was therefore made of extraction under greatly diminished pressure and hence lower temperature in much the same way as has been used so successfully by Dudley in his work on the pituitary principles. The temperature of the bath surrounding the boiling flask was from 45–50° and the internal temperature about 10° lower. It was found that the proline was readily extracted under these conditions and that it could be obtained in a satisfactorily pure condition. The solid amino-acids separating out from the butyl alcohol extraction carried out under reduced pressure contained a considerable amount of hydroxyproline but much was left in the aqueous solution long after all the proline had been removed. It appeared, therefore, that a more complete extraction of the hydroxyproline might be obtained through following up the butyl alcohol treatment by extraction with an alcohol with a greater capacity for mixing with the aqueous phase. Normal propyl alcohol was found to be satisfactory for the purpose although on prolonged extraction under reduced pressure much water was removed from the aqueous layer. The separation of the hydroxyproline from the other amino-acids thus extracted was a problem of great difficulty which was only partially solved.

The details will be given in a later section. It will be recalled that normal propyl alcohol and pure water are miscible in any proportion at room temperature but the high concentration of amino-acids in the aqueous solutions reduces the solubility of propyl alcohol to such an extent that it can be readily employed as a partially miscible solvent under the conditions of the present experiments.

In the following sections dealing with the individual amino-acids frequent reference is made to the use of Fischer's ester method for the isolation of amino-acids. In every case the admirable modification due to Levene and Van Slyke depending on the use of barium oxide and hydroxide for the liberation of the esters was employed. In the writer's experience the results of this method are superior to any of the others and the non-esterified portion is readily recovered and again treated with alcohol and hydrochloric acid. This repetition of the esterification was invariably carried out in the present work. As will be shown later, large amounts of hydroxyproline are obtained in the ester fraction when working with the above method. Hitherto it was believed that hydroxyproline was confined to the residues left after extraction of the esters with ether.

Individual Amino-Acids.

Glycine.—The first estimation of glycine was made as follows. Hydrolyzed gelatin, equivalent to 105 gm., was prepared as previously described, and the solution was extracted for about 100 hours with butyl alcohol at ordinary pressure. From the solid extracted amino-acids which contained alanine, leucine, and phenylalanine 4.0 gm. of glycine were obtained as the ethyl ester hydrochloride (7.45 gm.) by Fischer's method. The bulk of the glycine was left unextracted in the aqueous layer. This was diluted (2 liters), boiled under reduced pressure to remove dissolved butyl alcohol, and then the bases were removed with phosphotungstic acid in sulfuric acid (5 per cent) solution. After the phosphotungstates had been filtered off and washed, the filtrate was made alkaline with barium hydroxide, warmed for some time to expel residual traces of ammonia, and again filtered. The filtrate contained the barium salts of glycine, some hydroxyproline, and the di-basic acids. The latter were approximately separated by precipitating the concentrated solution (150 cc.) with 95 per cent alcohol (1,500 cc.). The precipitated barium salts contained practically the whole of the di-basic acids together with a considerable amount of glycine. The barium salts, after being washed with alcohol were dissolved in water, the barium was removed exactly with sulfuric acid, and the aspartic and glutamic acids (7 gm.) were slowly crystallized out at −10° from about 20 cc. of solution. The alcoholic mother liquor from the barium salts was concentrated and the barium removed exactly with sulfuric acid. It was then added to the mother liquor from the di-basic acids. The solution thus obtained contained 8 gm. of nitrogen and was very rich in glycine. It was hoped that a good separation of the glycine as picrate could be obtained by using Levene and Beatty's method. 100 gm. of picric acid were used and the concentrated solution was kept for 24 hours at −10°. Only 34.5 gm. of glycine picrate were obtained, which on decomposition gave 14.0 gm. of pure glycine. The separation, however, was very incomplete. On removing the picric acid from the filtrate in the usual way and then converting the residual amino-acids into ethyl esters by repeated treatment with alcohol and hydrochloric acid and liberating the pure esters by Levene and Van Slyke's method, a good yield of ethyl ester boiling under 65° at 10 mm. pressure was secured. The higher boiling fraction (11 gm.) was rejected. On hydrolysis 10.9 gm. of glycine were obtained, and on repeating the esterification the non-esterified residues gave a further 1.2 gm. of glycine. It should be noted that in spite of the high glycine concentration, only a small portion of it crystallized out as the ester hydrochloride. The presence of hydroxyproline appears to have considerable influence in preventing its crystallization. The total glycine thus separated from 115 gm. of gelatin is 30.1 gm. obtained as follows: 4.0 gm. from the amino-acids extracted by butyl alcohol, 14.0 gm. recovered as picrate, and 12.1 gm. obtained through the ester method. This is equivalent to 26.2 per cent of glycine. The mixed glycine thus separated showed no measurable optical activity in 5 per cent solution and contained 17.9 per cent of nitrogen compound with a calculated value of 18.6.

A second estimation of glycine was made in a much simpler fashion after it had been decided to abandon the use of picric acid. Gelatin (250 gm.) was hydrolyzed in the usual way and extracted with butyl alcohol at ordinary pressure for 5 days. The solid extracted amino-acids gave 19.5 gm. of ethyl ester hydrochloride equivalent to 10.5 gm. of glycine. The aqueous solutions after extraction were freed from bases with phosphotungstic acid as previously described and then esterified as before. Glycine ethyl ester hydrochloride equivalent to 11.2 gm. of glycine crystallized out and was worked up separately. On repeated esterification according to Levene and Van Slyke's method a total yield of 103 gm. of ethyl ester containing much alcohol was obtained, all of which boiled below 65° at 9 mm. pressure. Higher boiling esters (53 gm.) were rejected as before. The low boiling esters on hydrolysis gave 40.3 gm. of glycine, making a total yield of 62.0 gm. from 250 gm. of gelatin or 24.8 per cent. The mean of the two estimations is 25.5 per cent.

The foregoing experiments were completed before the extraction with butyl alcohol under reduced pressure had been employed. It is probable that the adoption of this procedure would be advantageous as under these conditions almost no glycine is extracted by the butyl alcohol and practically the whole of it is found in the aqueous residue.

Alanine.—40 gm. of amino-acids extracted by butyl alcohol at ordinary pressure from 125 gm. of hydrolyzed gelatin served as the material for estimating alanine. The mixed amino-acids were first crystallized from water and 9.4 gm. of sparingly soluble leucine and phenylalanine were removed in two crops. The mother liquor was concentrated and esterified in the usual way. Glycine ethyl ester hydrochloride (7.8 gm.) was filtered off and the esters were liberated and distilled. The ester fraction boiling below 90° at 9 mm. pressure was hydrolyzed with water and gave 10.9 gm. of amino-acid which were washed with alcohol and ether and dried. This product was fairly pure alanine as is seen from the following figures: N = 15.9, calculated 15.7; C = 40.2, calculated 40.4; H = 7.8, calculated 7.9 per cent. The alanine was apparently inactive in 2 per cent aqueous solution but the hydrochloride showed $[\alpha]_D = -9.9°$. It is probable that the only impurity in the alanine was a minute trace of glycine. The yield is equivalent to 8.7 per cent.

Isoleucine, Valine, and Aminobutyric Acid.—Isoleucine and valine are readily extracted by butyl alcohol under the conditions of the present experiments and, since alanine behaves in the same way, it is fair to assume that the intermediate homologue aminobutyric acid would show the same behavior. The possible presence of the latter acid has been indicated by Foreman, though the presence of isoleucine and valine in gelatin has been neither asserted nor denied. Accordingly large quantities of the extracted amino-acids from gelatin were subjected to systematic crystallization. Water was used as solvent for the various separations followed by progressively less dilute mixtures of methyl alcohol and acetone. Each fraction was examined for its nitrogen content by Van Slyke's gasometric method and, what was more important, its specific rotation was observed in neutral, acid, and alkaline media. While the complete separation of valine or isoleucine from their homologues is a matter of difficulty, their high specific rotations render their detection, when dealing as in the present case with exclusively unracemized products, a relatively simple matter. No fraction was obtained having a higher specific rotation in hydrochloric acid than that of ordinary leucine nor was any indication obtained of any acid intermediate between leucine and alanine. The reproduction of the detailed experimental data relating to the fractionation of the amino-acids appears unnecessary since the figures have no permanent value and are simply of negative significance. It is concluded, therefore, that isoleucine and valine are definitely absent from the gelatin molecule, while no indication has been obtained of the presence of aminobutyric acid.

Leucine and Phenylalanine.—Most of the leucine and phenylalanine is easily recovered by simple recrystallization from water or dilute methyl alcohol of the amino-acids extracted by butyl alcohol. By careful crystallization a succession of crops is easily obtained and, by carrying out amino nitrogen analyses on a few milligrams of each crop, it is easy to tell when the lower homologues begin to separate. In an actual experiment the following results were obtained by crystallizing 54 gm. of amino-acids obtained from 170 gm. of gelatin.

Crop I. 7.02 gm. N = 10.30 per cent; $[\alpha]_D = -11.5°$ in water, + 15.0° in HCl (20 per cent).

Crop II. 5.70 gm. N = 10.8 per cent; $[\alpha]_D = -12.0°$ in water, + 14.5° in HCl.

Crop III. 2.73 gm. N = 13.5 per cent; $[\alpha]_D = -6.25°$ in water, + 5.0° in HCl.

Crop III on recrystallization gave 0.95 gm. of the same composition as Crop II to which it was added. A small additional amount of the two amino-acids was recovered from the mother liquors by means of the ester method. The esters distilling below 80° at 11 mm. pressure were removed and the higher boiling residue without distillation was saponified by boiling with water and fractionally crystallized from water. 0.8 gm. of mixed phenylalanine and leucine in almost equal proportions, as judged by elementary analysis, was thus recovered from the least soluble fraction, while the mother liquors contained much hydroxyproline and some serine. The total yield of phenylalanine and leucine from 170 gm. of gelatin was 14.47 gm. corresponding to 8.5 per cent. Two other similar experiments gave results between 7 and 8 per cent.

The amount of leucine in the mixture far exceeds that of phenylalanine. Judged by analysis the proportion is about 5 : 1. On simple recrystallization from water, perfectly pure leucine with optical properties closely corresponding to those obtained by Ehrlich is readily obtained (C = 54.8; H = 9.8; N = 10.7 per cent).

The complete separation of phenylalanine from leucine proved to be a difficult matter. Crystallization of the hydrochlorides and other salts was ineffectual, while experiments aiming at the preparation of nitro or sulfonic acid derivatives were only partially successful. Finally recourse was had to the application of the conventional ester method applied to the leucine and phenylalanine fraction from which as much leucine as possible had been removed by crystallization. The ester soluble in water but extracted by ether gave fairly pure phenylalanine but the yield never exceeded 1.4 per cent of the gelatin. This figure agrees fairly closely with estimates of inactive phenylalanine from racemized gelatin already recorded by the writer.

From the above experiments it is inferred that phenylalanine and leucine together comprise 8.5 per cent of the amino-acids from gelatin and that they are approximately in the proportion of 7.1 per cent leucine and 1.4 per cent phenylalanine. A similar experiment in which the leucine and phenylalanine were extracted by butyl alcohol under reduced pressure gave a yield of 8.2 per cent for the mixed acids.

The absence of valine and isoleucine together with the low proportion of phenylalanine undoubtedly makes gelatin a desirable substance for the easy preparation of pure active leucine. While the yield is not high, the absence of isomers greatly facilitates its separation, if extraction with butyl alcohol is employed as a preliminary step.

Tyrosine.—Tyrosine is not ordinarily regarded as a constituent of gelatin but traces of it were encountered in all the specimens of gelatin examined. In two cases sufficient was present to furnish a few mg. of the pure substance but in every case sufficient was present to give strong reactions with bromine water, Millon's reagent, and diazobenzenesulfonic acid. The tyrosine was readily extracted by butyl alcohol and showed some tendency to remain in solution, for it was recovered from the copper salts, sparingly soluble in alcohol, separated from the crude proline fraction. Its amount was certainly not in excess of a fraction of 0.1 per cent and the amount separated was even less, so that it is probably a safe deduction that tyrosine is not an integral part of the gelatin molecule. The unsatisfactory methods of purifying gelatin would undoubtedly account for the presence of traces of it as an impurity.

Proline.—As already stated in the earlier part of the present communication, it was found that extraction of hydrolyzed gelatin with butyl alcohol at normal pressure, while effective in removing the whole of the proline, gave an impure product owing to the presence of large quantities of diketopiperazines. A considerable proportion of these cyclic anhydrides appeared to be formed as a secondary reaction during the prolonged boiling of proline in butyl alcohol solution with other amino-acids. For this reason extraction under diminished pressure and hence lower temperature was adopted. The apparatus used is shown in Fig. 1 and requires almost no description. It is a slight modification of that used by Dudley and should be of value for other purposes. An efficient water pump attached to the apparatus maintained an average pressure of about 10 mm. The water bath for heating the Claisen flask used as the extraction vessel was maintained at 45–50°.

The hydrolyzed gelatin was placed in the side-necked bottle shown on the left. When once the extraction is started and the heating adjusted the apparatus can safely be left for 24 hours at a time. The extraction of the proline from 100 gm. of hydrolyzed gelatin in a volume of about 200 cc. in the apparatus shown, working at moderate speed, required about 60 hours. Solid amino-acids soon appeared in the extraction flask and were comprised chiefly of leucine with some phenylalanine and hydroxyproline. It is well to change the flask and start with a fresh supply of alcohol at daily intervals so that the separation of solid amino-acids does not cause undue bumping, and in addition the likelihood of bringing about changes in the proline is reduced. At the end of the extraction, the combined butyl alcohol extracts are filtered for suspended amino-acids and the latter washed with

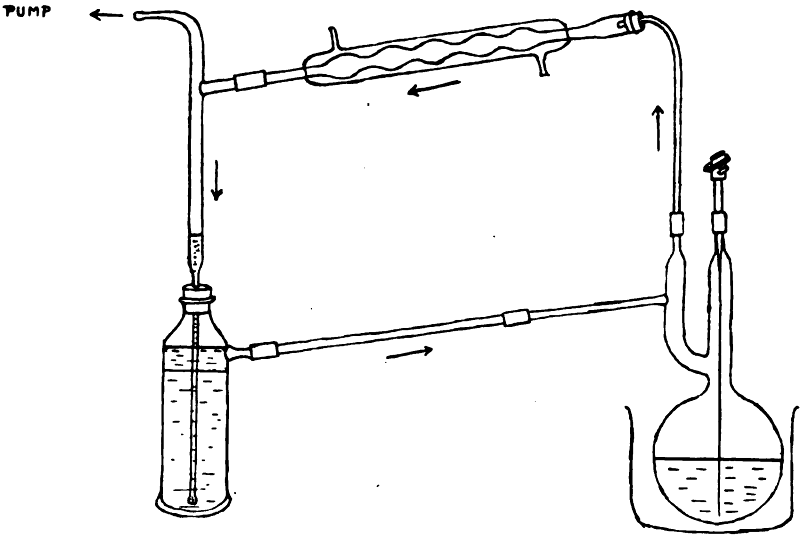


FIG. 1.

a little more of the alcohol. The filtrate was then evaporated to syrup under reduced pressure, dissolved in absolute alcohol, again evaporated, and redissolved in 50 cc. of absolute alcohol. Only a minute trace of solid separated on long standing at a low temperature and this was filtered off. Alcohol was removed from the filtrate by evaporation and the clear syrup dissolved in water. The specific rotation of the product without further purification observed in aqueous solution was −78°, a figure which closely approximates that of pure proline. From the experiences gained in previous experiments, it was decided that purification by way of the copper salts, or by precipitation with phosphotungstic acid was of negligible value. Recourse was therefore had to treatment with mercuric acetate and baryta as previously described. Relatively little impurity was removed by this treatment and the proline was recovered from the filtrate, in the manner already referred to, evaporated to dryness, dissolved in alcohol to remove a trace of insoluble inorganic impurity, and then evaporated and dried under reduced pressure.

The proline thus obtained had only a light yellowish tinge, and crystallized slowly but completely in the desiccator. The amount of amino nitrogen in the purified proline fraction from 100 gm. of gelatin, as determined by Van Slyke's method, never exceeded 10 mg. and for this amount a suitable correction was applied. The specific rotation of the product varied from 79.3–82.5°. On elementary analysis good results were obtained (52.0 to 52.5 per cent C; 7.8 to 7.95 per cent H; 12.2 to 12.4 per cent N), though as previously mentioned these results are of less value in judging of the purity of the product than the estimations of optical activity. The proline thus separated gave yields of the ethyl ester, copper salt, and proline hydantoin comparable with those obtainable from the pure substance. Apparently the only impurity that might be present in significant amounts in the proline obtained as described above is some diketopiperazine with properties allied to those of proline. To guard against this, exceptional care was taken that the hydrolysis of the gelatin was as complete as possible, and the proline when isolated was heated under pressure with hydrobromic acid at 130° in order to hydrolyze any diketopiperazines and thus lead to the detection of other amino-acids. Such acids, especially hydroxyproline, were invariably found when the extraction of the proline had been carried out at ordinary pressure, but when the extraction was performed as described in the present section they were not detected. The following yields of proline, corrected for a trace of non-amino nitrogen, and calculated on a content of 12.2 per cent non-amino nitrogen, were obtained. This method of calculation is preferable to direct weighing owing to the hygroscopic character of the substance.

150 gm. gelatin gave 13.9 gm. proline = 9.2 per cent.
100 " " " 10.5 " " = 10.5 " "
80 " " " 7.02 " " = 8.6 " "
200 " " " 19.2 " " = 9.6 " "
125 " " " 11.8 " " = 9.5 " "

The average of the above results gives 9.5 per cent of proline as the estimated yield. It is probable that the method outlined above represents the simplest method for the preparation of optically active proline.

Hydroxyproline.—This substance it will be recalled was isolated from gelatin by Fischer through working up the residue left after the separation and extraction of the ordinary amino-acid esters according to his well known method. A yield of about 3 per cent was obtained and the same method has been followed by Leuchs and other workers. It was generally assumed, though not specifically stated, that either hydroxyproline did not form an ester under the customary conditions, or, if it did so, the ester was not extracted by ether. On this assumption, it was thought that the separation of hydroxyproline from the other amino-acids of gelatin which had previously been partially separated by the use of solvents would be a relatively easy matter. This was soon found not to be the case for the simple reason that the larger part of the hydroxyproline was found to yield an ester readily which was easily extracted by ether under the conditions employed.

As has already been stated a considerable part of the hydroxyproline in hydrolyzed gelatin may be extracted by butyl alcohol and is found among the amino-acids which separate from solution in the extraction flask. This material, therefore, seemed promising for preliminary attempts to separate the hydroxyproline

20 gm. of this material were esterified in the usual way and the ester was liberated by Levene and Van Slyke's method and extracted by ether. The fraction of ester boiling below 100° was rejected and the residual ester without distillation was at once hydrolyzed with boiling water. On crystallizing from water, a small crop of leucine and phenylalanine separated first, followed by a very soluble substance which was expected to be mainly serine. But it had the following composition: 47.4 per cent C; 7.4 per cent H; 10.9 per cent N. The amino nitrogen was only 3.4 per cent and the specific rotation in water −53.5°. These results clearly indicated that the product contained a great deal of hydroxyproline and on crystallizing from 90 per cent methyl alcohol almost pure hydroxyproline was readily obtained (melting point 263°; 45.5 per cent C; 6.85 per cent H; $[\alpha]_D$ −72.0°; amino nitrogen 0.3 per cent). It was clear, therefore, that hydroxyproline is esterified under the customary conditions with alcohol and hydrochloric acid and that a large part of it is extracted by ether, using Levene and Van Slyke's method for the liberation of the esters.

The residues remaining after the liberation of the esters were freed from barium salts as usual and, after evaporation, hydrochloric acid was removed by successive treatments with lead hydroxide and silver oxide. The filtrate was freed from metals with hydrogen sulfide and a good deal of optically inactive material was removed by precipitation with mercuric acetate and baryta as applied in the purification of proline. The filtrate freed from barium and mercury salts gave on concentration crude hydroxyproline (melting point 265°; 46.0 per cent C; 6.8 per cent H; 10.8 per cent N; 0.7 per cent amino N; $[\alpha]_D$ in water −64.5°). On recrystallization from 90 per cent methyl alcohol pure hydroxyproline was readily obtained, and it was identical in every respect with the product obtained from the ester fraction. The amount of hydroxyproline obtained in a pure state from the ester and from the residues was only about 0.3 gm. in each case but the loss on purification is very great. But at least it was settled that about as much of the hydroxyproline passed into the ester fraction as remained behind and on repeated esterification the proportion was materially greater.

In addition to the hydroxyproline fractions just described which were obtained from the the amino-acids mixture extracted by butyl alcohol, variable amounts were isolated by substantially similar methods from the aqueous residue remaining from the butyl alcohol extraction. It was clear, therefore, that improved methods were necessary not only for the extraction of the hydroxyproline but for its isolation and purification and that but little assistance was likely to be derived from the ester method as previously used for the preparation of hydroxyproline.

As has been already stated, the extraction of the hydroxyproline from the hydrolyzed gelatin could be rendered much more complete by following up the first extraction with butyl alcohol under reduced pressure by extraction with propyl alcohol under similar conditions. This preliminary separation from the bases, di-basic acids, and most of the glycine was of great value but the difficulty now encountered was that of isolating and purifying the hydroxyproline from accompanying amino-acids. A large number of experiments have been made with this object in view, some of which were complete failures and others were partly successful. A few of these are summarized.

(*a*) Hydroxyproline, like proline, evolves little or no nitrogen when treated with nitrous acid as in Van Slyke's method. Since the greatest difficulty in purifying hydroxyproline consists in the removal of amino-acids containing the (NH_2) group, which reacts with nitrous acid, it was thought that possible advantage might be taken of this reaction. Experiments on mixtures containing hydroxyproline were made with sodium nitrite or amyl nitrite acting in acetic or sulfuric acid solution for various lengths of time up to several days and at temperatures varying from 15–100°. The unchanged amino-acids were recovered, after removal of any sulfuric acid by baryta, by precipitation with mercuric acetate and sodium carbonate according to the methcd described by Neuberg and Kerb. This method of precipitation is effective for hydroxyproline as was found by separate experiments. It was found that the action of nitrous acid was useless for the purpose in view. Unless a very large excess of nitrous acid was used a great deal of unchanged amino-acid persisted and when a large excess of nitrous acid was employed decomposition of the hydroxyproline took place presumably through the formation of a nitroso compound.

(*b*) Hydroxyproline is well known to be extremely soluble in water and sparingly soluble in alcohol. It was found that when present with the amino-acids extracted by solvents from hydrolyzed gelatin it could be separated conveniently from much of the latter in the following simple manner. The mixed acids are heated with an equal weight of water on the water bath and then, irrespective of whether complete solution has taken place, nine parts of methyl alcohol are added with shaking, and the mixture is allowed to stand for a day or so. The precipitated amino-acids are filtered off, washed with methyl alcohol, and analyzed for both total and amino nitrogen, in order to be sure that no hydroxyproline remains. With all the products thus far examined, the whole of the non-amino nitrogen of the hydroxyproline has been found in the filtrate together with variable but considerable amounts of soluble amino-acids, especially glycine, alanine, and serine. Some further separations of amino-acids from this hydroxyproline fraction can be secured by the following method.

(*c*) Reference has already been made to the use of mercuric acetate and barium hydroxide for the precipitation of impurities in the proline fraction. Proline is not precipitated under these conditions and similarly it was found that but little hydroxyproline was carried down with the precipitate. Most amino-acids derived from proteins are precipitated by this reagent but the lower members of the series are less completely removed than the higher ones. While the separation is incomplete, the fact that all the reagents employed are readily removed without introducing other substances makes the process of some value. The following experiments illustrate its use:

(1) 1 gm. of hydroxyproline containing about 7 per cent of impurity as judged by its specific rotation and amino nitrogen content was treated with mercuric acetate (20 cc. of saturated solution) and barium hydroxide (80 cc. of saturated solution). From the filtrate on removal of mercury and barium salts 0.87 gm. of hydroxyproline $[\alpha]^{20}_{D} = -72.5°$ was recovered.

(2) 50 cc. of a solution containing 0.631 gm. of amino nitrogen mostly as glycine, alanine, and serine and 0.280 gm. of non-amino nitrogen as hydroxyproline were treated with mercuric acetate (60 cc.) and crystallized baryta (60 gm.). The filtrate after removal of mercury and barium contained 0.276 gm. of amino nitrogen and 0.251 gm. of non-amino nitrogen, showing that rather more than half of the amino-acids had been removed with an apparent loss of about 10 per cent in hydroxyproline.

(*d*) It has been shown by Levene and Van Slyke that proline and hydroxyproline unlike most other amino-acids do not form readily crystallizable picrolonates. It was thought that possible use might be made of this fact for the further separation of hydroxyproline from amino-acids, but when other than very simple mixtures of pure substances were used the amount of purification effected was slight and on the whole the use of picrolonic acid was less effective than the preceding method (*c*).

(*e*) The conversion of proline into a uramino-acid by the action of potassium cyanate, and its subsequent transformation into proline hydantoin has already been described as a useful method for the characterization of proline. Since so few attractive derivatives of hydroxyproline are known it appeared worth while to make similar experiments with this substance.

Hydroxyproline (3 gm.) in 15 cc. of water was allowed to stand in a warm place for a few hours with potassium cyanate (3 gm.). An additional

0.5 gm. of the cyanate was added and the mixture was heated on the water bath for 1 hour. It was then made just acid to Congo red with sulfuric acid, diluted to 50 cc., and extracted for 8 hours with ether in a continuous extractor. As with proline but unlike most amino-acids other than glycine and serine only a trace of substance (0.09 gm.) was extracted.

The uramino-acid remaining in the aqueous solution was converted into the hydantoin by evaporation on the water bath with 30 cc. of hydrochloric acid. The solution was then reextracted with ordinary ether. The hydroxyproline hydantoin is taken out rather slowly. After 24 hours, 3.0 gm. were obtained while an additional 0.55 gm. was obtained by continuing for 12 hours more. The substance is very soluble in water, alcohol, and ether and fairly soluble in chloroform. It is best crystallized either by dissolving in a drop or two of alcohol and then adding benzene in which it is almost insoluble or by slow evaporation of its aqueous solution. The substance crystallizes in needles and melts rather indefinitely at 162–165°.

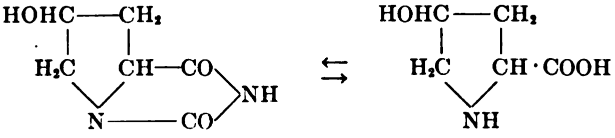
Analysis. 0.1520 gm. gave 0.2564 CO_2 and 0.0725 H_2O.
0.1425 " " 0.0255 N(Kjeldahl).

	C *per cent*	H *per cent*	N *per cent*
Calculated for $C_6H_8N_2O_3$	46.2	5.13	17.9
Found	46.0	5.30	17.8

γ-Hydroxyproline hydantoin is strongly levorotatory in aqueous solution though much less so than prolylhydantoin.

$$C = 1.50;\ l = 2.0;\ \alpha = -\ 2.92^\circ$$
$$[\alpha]_D^{20} = -\ 07.2^\circ$$

The yield of hydantoin from pure hydroxyproline was so good and its ready solubility in ether and water distinguished it from so many related substances that it appeared likely that use could be made of it for the characterization of hydroxyproline. Accordingly experiments were made to determine whether the hydantoin could be reconverted into hydroxyproline. Prolonged boiling (36 hours) with aqueous barium hydroxide (10 per cent) was found to be the best method. The evolution of ammonia and hence the progress of hydrolysis is conveniently followed by means of a bulb trap placed at the top of the reflux condenser containing a little alizarin and known amounts of standard acid. After no more ammonia is evolved, the barium salts are removed exactly with sulfuric acid and the acid is recovered by concentration and precipitation with alcohol. As is well known, optically active hydantoins readily undergo racemization on treatment with alkali, probably owing to tautomeric changes involving a loss of asymmetry of the α carbon atom. Hydroxyproline and its hydantoin, however, contain 2 asymmetric carbon atoms, hence it was not surprising that the hydroxyproline recovered from the hydantoin was the partially racemic γ-hydroxyproline of Leuchs and Bormann (1919) and possessed a specific rotation of − 9.2°.



The yield of hydroxyproline recovered from the hydantoin varied from 64 to 73 per cent of the theoretical amount so that considerable losses are involved in the reconversion.

In the light of the foregoing experiments with hydroxyproline, the following attempt to estimate the hydroxyproline in hydrolyzed gelatin was carried out.

Hydrolyzed gelatin (100 gm.) was extracted for 72 hours under reduced pressure with butyl alcohol and the alcohol-soluble proline removed as previously described. The amino-acids (9.5 gm.), chiefly leucine, accompanying the proline contained a little hydroxyproline and to recover this the product was treated with 90 per cent methyl alcohol as described under the preceding section, (*b*). The filtrate containing the hydroxyproline was removed and later added to the extract obtained by continuing the original extraction for a further 4 days with propyl alcohol. The volume of the aqueous hydrolyzed gelatin solution fell during the last extraction from 215 to 120 cc. but practically all the bases and dicarboxylic acids remained unextracted. The aqueous solution contained 12.01 gm. of total nitrogen, of which 7.16 gm. were not precipitable by phosphotungstic acid and of this only 0.71 gm. was non-amino nitrogen. The last 12 hours extraction with propyl alcohol extracted only a fraction of a gram of solid substance.

The propyl alcohol extracts were combined with the methyl alcohol filtrate above referred to and the alcohols were then removed by evaporation under reduced pressure. The residue was dissolved in water and made up to 250 cc. This solution, it is believed, contains practically the whole of the hydroxyproline together with a large amount of lower amino-acids especially alanine, serine, and some glycine. The proportion of amino to non-amino nitrogen was about 2 : 1. A certain amount of purification was effected as follows: The solution was acidified with sulfuric acid and precipitated with phosphotungstic acid.

A minute amount of oily precipitate was removed and assurance was thus obtained of the complete removal of the bases. The filtrate was then freed from sulfuric acid and precipitated as described under (*c*) with mercuric acetate (300 cc. of saturated solution) and barium hydroxide (30 gm.). The filtrate, after quantitative removal of barium and mercury salts, was concentrated under reduced pressure. The solution (100 cc.) contained 1.51 gm. of non-amino nitrogen and 1.38 gm. of amino nitrogen. It was strongly levorotatory showing $\alpha = -14.96°$ in a 1 dm. tube. Assuming that the whole of the non-amino nitrogen was in the form of hydroxyproline, the concentration of the latter would be 14.1 gm. corresponding to a rotation of about $-10.6°$. It would therefore appear that other levorotatory amino-acids were present and of these the presence of serine is almost certain.

On carefully crystallizing the above solution by slow evaporation in the desiccator large amounts (7.2 gm.) of hydroxyproline were obtained, but after the removal of one or two crops had been effected the products became progressively less pure and no further separation was possible.

After as much as possible of the hydroxyproline had been separated by direct crystallization, the mother liquors were treated with potassium cyanate as precisely described in Section (*e*). In this way a considerable yield of hydroxyproline hydantoin, soluble in ether, was obtained showing a specific rotation of $-89°$. On hydrolysis of the hydantoin with baryta, an additional 5.2 gm. of partially racemic hydroxyproline were obtained, making a total of 12.4 gm. (C = 45.5 per cent; H = 6.98 per cent). The amount of amino nitrogen in the product was 0.8 per cent. If one takes into consideration the known experimental losses of the preceding manipulations, one may conclude that the deductions drawn from analytical and optical rotations recorded in the preceding paragraph, indicating the presence of 14.1 per cent of hydroxyproline, are approximately correct.

Aspartic and Glutamic Acids.

The separation of the greater part of these acids was very simply accomplished as described in the section dealing with glycine. Hydrolyzed gelatin was thoroughly extracted with butyl alcohol, the aspartic and glutamic acids together with the bases and much glycine remaining in the aqueous solution. The bases were removed from the solution by precipitation with phosphotungstic acid in the usual fashion and the filtrate after removal of sulfuric and phosphotungstic acids was made strongly alkaline with barium hydroxide. If 100 gm. of gelatin were used, an excess of about 30 gm. of crystallized barium hydroxide was used. The concentrated solution of barium salts (150 cc.) was added by degrees with shaking to 95 per cent alcohol (1,500 cc.). The precipitated barium salts containing the aspartic, glutamic, and other di-basic acids if present, together with some glycine, were washed with alcohol, dissolved in water, and the barium was exactly removed with sulfuric acid. On concentrating the solution and allowing it to stand for several days at a very low temperature (−10°), the bulk of the acids readily crystallized out. The yield of mixed aspartic and glutamic acids thus obtained varied from 5.6 to 7.1 per cent. An additional amount of the mixed acids was obtained by converting the amino-acids in the filtrate into their esters. The fraction boiling below 100° at 5 mm. pressure was rejected and the remaining esters without distillation were hydrolyzed by boiling with barium hydroxide solution. The barium was then removed with sulfuric acid. The acid solution was then neutralized with sodium hydroxide and treated alternately with silver nitrate and sodium hydroxide as long as a white precipitate was obtained. The method used was that employed by the writer for the precipitation of silver β-hydroxyglutamate. The precipitated silver salts were filtered off, washed with water, and decomposed with hydrogen sulfide. The filtrate on concentration gave a further yield of crystalline aspartic and glutamic acids amounting to 2.1 to 2.5 gm. The total yield of di-basic acids in the best experiment amounted to 9.2 per cent.

The mixed acids gave results on analysis indicating a mixture of about five parts of glutamic acid to three of aspartic acid. This result was confirmed by separation of the glutamic acid as hydrochloride in the usual fashion while in another portion of the mixed acids barium aspartate was crystallized out from saturated baryta solution. (Independent solubility determinations showed that under these conditions in an ice box at about 3° the solubility of active barium aspartate is equivalent to 0.5 gm. of aspartic acid per 100 cc. of saturated baryta solution. The solubility of the optically inactive salt is materially less.) Finally molecular weight determinations of the mixed acids by titration, using litmus as indicator, gave results closely in accord with the above. As a mean of the most satisfactory experiments, it is concluded that gelatin contains close to 3.4 per cent of aspartic acid and 5.8 per cent of glutamic acid.

It should be noted here that a careful search was made for β-hydroxyglutamic acid which had already been found in casein, gliadin, and glutenin. It would have been found in the alcohol-insoluble barium salts if present but none could be detected. Moreover the total acidity, to litmus, of the solution of the mixed acids obtained from the barium salts was so nearly equivalent to the isolated quantity of aspartic and glutamic acids that it is probably safe to conclude that no significant amount of di-basic acid other than glutamic and aspartic acids is present in gelatin.

Serine.—The difficulties of separating serine with existing methods from the products of hydrolysis of gelatin are very great. The serine mainly accompanies the hydroxyproline and on esterifying is found in the high boiling fraction of esters along with the latter substance. The separation of the two acids has proved so far insurmountable, though, owing to its large quantity, much hydroxyproline could be obtained from the mixture by direct crystallization. From the mother liquors it is possible to separate as picrolonate large amounts of an acid approaching serine in composition and general properties, but its optical rotation never coincided exactly with those of pure levo serine as prepared by Fischer and Jacobs. In aqueous solution figures between −10° and −12° are obtained but unlike pure *l*-serine it was usually feebly levorotatory in hydrochloric acid solution. The difficulties of crystallizing active serine are well known and so far none of the various derivatives tried has proved satisfactory. The writer's impression is that gelatin contains relatively large amounts of serine and possibly homologues of it in addition, but until improvements in the methods of separation are available a final decision cannot be reached. The figure 0.4 per cent for serine given by Fischer, which is manifestly far too low, is therefore retained for the present.

Histidine, Arginine, and Lysine.—New determinations of these bases appeared unnecessary, but frequent confirmation has been obtained of the existing figures furnished by Van Slyke's analyses. The earlier figures of Kossel and Kutscher and of Hart while agreeing well for arginine appear somewhat low for lysine.

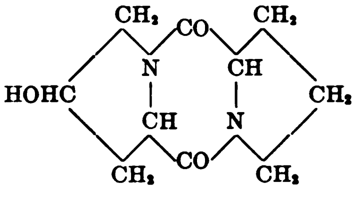
DISCUSSION OF RESULTS.

The results of the preceding analysis of the products of the hydrolysis of gelatin are collected in Table I together with the main results of other workers. As regards the new results attention may be drawn to the high proportion of glycine and the very much larger amount of alanine than has been recorded hitherto. The figure given for hydroxyproline (14.1 per cent) may prove too high as it is based partly on analytical and polarimetric data. As previously stated complete purification of the hydroxyproline was not accomplished but at least 7 per cent was obtained optically and analytically pure and the presence of much more in the mother liquors was certain. A large part of the latter was isolated in the partially racemic form by conversion into the hydantoin and subsequent hydrolysis. The amounts of proline and leucine accord well with those found by Skraup and von Biehler. The new results for aspartic are considerably higher than those previously found. The figure given by Skraup and von Biehler for glutamic acid (16.8 per cent) is extraordinarily high and certainly no such amount was present in the product examined by the writer. It would appear possible that glycine hydrochloride, which crystallizes readily from concentrated hydrochloric acid, may have contaminated the glutamic acid hydrochloride, and moreover the glycine found by Skraup and von Biehler is undoubtedly too low. The yield of serine recorded (0.4 per cent) is taken from Fischer's analysis and also is undoubtedly much too low. The complete separation of serine and hydroxyproline was not accomplished. In addition to the substances recorded in the table, a significant amount of unidentified sulfur compounds has still to be accounted for.

TABLE I.

Amino-acid.	Dakin.	Fischer, Levene, and Aders; Hart; Kossel and Kutscher.	Levene and Beatty.	Skraup and von Biehler.
	per cent	*per cent*	*per cent*	*per cent*
Glycine	25.5	16.5	19.25	12.4
Alanine	8.7	0.8	3.0	0.6
Aminobutyric acid	None found.			
Valine	None.	1.0		
Isoleucine	"			
Leucine	7.1	2.1	6.75	9.2
Serine	0.4	0.4		
Phenylalanine	1.4	0.4		1.0
Tyrosine	0.01			
Proline	9.5	5.2	6.25	10.4
Hydroxyproline	14.1	3.0	6.4	3.0
Aspartic acid	3.4	0.6		1.2
Glutamic "	5.8	0.9	1.75	16.8
Hydroxyglutamic acid	None.			
Histidine	0.9	0.4		0.4
Arginine	8.2	7.6		9.3
Lysine	5.9	2.8		6.0
Ammonia	0.4	0.4		0.4
Total	91.31	42.1		70.7

Hydroxyprolylproline Anhydride.



This substance was repeatedly encountered in the crude proline fraction obtained by extracting hydrolyzed gelatin with butyl alcohol under ordinary pressure. The substance has a certain special interest as representing the first peptide to be obtained containing the hydroxyproline nucleus and also it is apparently unique in containing three rings. Experiments made with gelatin that had been partially hydrolyzed at room temperature with 70 per cent sulfuric acid for about a week gave unsatisfactory results and it appears that the most suitable material for its preparation is obtained by boiling gelatin with dilute sulfuric acid as described on page 500, omitting the heating in an autoclave. Sulfuric acid is then removed as usual and the concentrated neutral solution maintained at about 60° is then extracted for 2 or 3 days with butyl alcohol under ordinary pressure. The extract filtered from insoluble amino-acids is evaporated under diminished pressure and dissolved in ten parts of absolute alcohol. The filtered alcoholic solution is then evaporated and the residue dissolved in a little water and clarified with charcoal is allowed to crystallize slowly in a desiccator. Under favorable conditions a considerable separation takes place of irregular prismatic crystals from the syrupy mother liquor. These crystals are drained as much as possible by spreading on porous tiles and then purified as follows: 5 gm. of the crystalline magma are dissolved in 20 cc. of absolute alcohol and then 100 cc. of dry ether are gradually added. A yellow sticky mass containing much proline and a few crystals separates promptly and is rejected. The mother liquor on evaporation at room temperature gives beautiful large prisms of the new substance. The crystals are dried on a plate and for analysis are recrystallized from a little water in which it is extremely soluble. The yield of peptide anhydride varies from a trace to as much as 2.75 per cent of the gelatin taken.

Analysis. 0.1500 gm. gave 0.3141 gm. CO_2 and 0.0905 gm. H_2O.
0.2059 " " 0.0272 N (Kjeldahl).

	C *per cent*	H *per cent*	N *per cent*
Calculated for $C_{10}H_{14}N_2O_3$	57.1	6.67	13.3
Found	57.1	6.69	13.2

The substance is very readily soluble in water and alcohol but sparingly soluble in pure ether and insoluble in most other organic solvents. Its aqueous solution reacts very feebly acid to litmus and on boiling with precipitated copper oxide no blue salt is formed. It does not react with bromine water or formaldehyde, and gives no nitrogen with nitrous acid. Millon's reagent gives neither a precipitate nor color. Silver nitrate alone or with ammonia gives no precipitate but on boiling slow reduction occurs. Concentrated solutions (1 per cent and over) give an orange color on treatment with diazobenzenesulfonic acid and sodium carbonate. Phosphotungstic acid partially precipitates it from strong solutions, the crystalline phosphotungstate readily dissolving in excess of the reagent. Picric acid produces no precipitate.

The optical rotation is notably high and was observed in aqueous solutions.

$$C = 1.0 \qquad l = 2.0 \qquad \alpha = -2.84^\circ$$

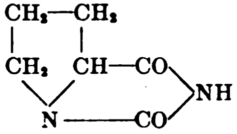
$$[\alpha]^{20}_{D} = -142.0^\circ$$

The melting point of the product appears to be variable. When crystallized from ether it melts sharply at 102–103° while when crystallized from water it melts at 135–140°. This phenomenon was repeatedly observed but whether it is due to some form of isomeric change remains undecided.

That the substance isolated as above described was actually hydroxyprolylproline anhydride possessing the formula given above and not the isomeric prolylproline was proved by hydrolysis. 3 gm. of the peptide were heated in a sealed tube with 9 cc. of fuming hydrobromic acid for 4 hours at 130°. The tube was then opened, the contents were diluted with water, and evaporated under reduced pressure as far as possible. The residue was dissolved in water (20 cc.) and vigorously shaken with excess of freshly precipitated silver oxide until the reaction was neutral to Congo red. The silver bromide was filtered off and silver removed from the filtrate with hydrogen sulfide. On concentration a completely crystalline mass was obtained which was readily separated into soluble proline and insoluble hydroxyproline on treatment with absolute alcohol.

The hydroxyproline weighed 1.40 gm., melted at 263°, and contained no amino nitrogen. On analysis it gave 46.1 per cent of carbon, 6.83 per cent of hydrogen, and 10.8 per cent of nitrogen. Its specific rotation in water ($C = 0.93$) was -74.8°. The proline fraction weighed 1.72 gm. and on treatment with cold absolute alcohol a trace more of hydroxyproline was separated. On analysis it gave 52.0 per cent of carbon, 7.98 per cent of hydrogen, and 12.0 per cent of nitrogen. Amino nitrogen was absent. The proline was partially racemized by the treatment with hydrobromic acid, for its specific rotation in water was only −48° and on converting it into the copper salt in the usual fashion, part was soluble in methyl alcohol while the insoluble portion, on drying, gave the characteristic purple-violet copper salt of inactive proline.

Inactive Proline Hydantoin.



Optically active proline hydantoin has frequently been made use of by the writer for the identification of proline. The inactive derivative has not hitherto been described. It was prepared as follows. Gelatin (100 gm.) was heated in an autoclave at 130° for 10 hours with crystallized barium hydroxide (250 gm.) and water (500 cc.). The mixture was neutralized with sulfuric acid, filtered, and concentrated and then extracted for 36 hours with butyl alcohol under diminished pressure as already described. The filtered extract was evaporated to dryness and taken up in absolute alcohol. The crude proline solution was then purified with mercuric acetate as already described for the active product. The partially purified yield of proline which was entirely inactive amounted to 4.2 gm. 2 gm. of this product was greatly warmed on the water bath for 1 hour with 2.0 gm. of potassium cyanate and 10 cc. of water. The residue was taken up in water and made just acid to Congo red with dilute sulfuric acid, and then extracted for about 6 hours with ether to remove traces of contaminating uramino-acids. The aqueous solution was then evaporated with 10 cc. of concentrated hydrochloric acid to convert the remaining uramino-acid into the hydantoin. On prolonged reextraction with ether *r*-proline hydantoin was obtained in a yield of 1.8 gm. It is extremely soluble in water and alcohol, readily soluble in ether and acetone, and sparingly soluble in chloroform. It crystallizes from its concentrated aqueous solution in the form of highly refractive apparently hexagonal prisms. It melts at a distinctly lower temperature than the active variety; namely, 142–143°.

Analysis. 0.1532 gm. gave 0.2881 gm. CO_2 and 0.0801 gm. H_2O.

Calculated for $C_6H_8N_2O_2$: 51.4 per cent C; 5.71 per cent H.
Found: 51.3 per cent C; 5.80 per cent H.

SUMMARY.

An analysis of the products of hydrolysis of gelatin has been made making use of extraction with butyl alcohol for the separation of certain groups of amino-acids.

Butyl alcohol extraction under ordinary pressure readily removes the alanine, leucine, and phenylalanine, while hydroxyproline and serine are extracted more slowly. Glycine is extracted to a relatively small extent.

The extraction of proline is best accomplished by extraction with butyl alcohol under reduced pressure, thus limiting secondary changes. Propyl alcohol may be used to complete the extraction of the hydroxyproline. Various methods are described for the separation and purification of proline and hydroxyproline together with some new derivations. It was found that hydroxyproline is readily esterified and that much of it passes into the ester fraction soluble in ether when liberated with barium hydroxide. Reasons are advanced for the belief that gelatin may yield as much as 14 per cent of hydroxyproline.

The strong acids and bases from gelatin are not extracted by butyl alcohol. The former consist entirely of aspartic and glutamic acids. The absence of hydroxyglutamic acid was established. The absence of valine and isoleucine was determined. Gelatin appears, therefore, a very suitable material for the preparation of optically pure leucine.

A new tricyclic peptide from gelatin is described; namely, γ-hydroxyprolylproline anhydride.

The preparation of the hydantoin deriveratives of *l*-hydroxyproline and *r*-proline is also recorded.

The quantitative results of the estimation of the amino-acids from gelatin are recorded in tabular form. The total thus estimated amounts to 91.31 per cent to which must be added considerable additional amounts of serine and possibly allied substances which could not be satisfactorily separated from the hydroxyproline. Unidentified sulfur derivatives are also present.

BIBLIOGRAPHY.

Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
Dakin, H. D., *Biochem. J.*, 1919, xiii, 398.
Dudley, H. W., *J. Pharmacol. and Exp. Therap.*, 1919, xiv, 295.
Fischer, E., *Ber. chem. Ges.*, 1902, xxxv, 2660.
Fischer, E., Levene, P. A., and Aders, R. H., *Z. physiol. Chem.*, 1902, xxxv, 70.
Leuchs, H., and Bormann, K., *Ber. chem. Ges.*, 1919, lii, 2086.
Levene, P. A., and Beatty, W. A., *Z. physiol. Chem.*, 1906, xlix, 252.
Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1909, vi, 419.
Maillard, L.-C., *Compt. rend. Acad.*, 1911, cliii, 1078.
Neuberg, C., and Kerb, J., *Biochem. Z.*, 1912, xl, 498.
Skraup, Z. H., and von Biehler, A., *Monatschr. Chem.*, 1909, xxx, 467.

A QUANTITATIVE METHOD FOR THE DETERMINATION OF VITAMINE IN CONNECTION WITH DETERMINATIONS OF VITAMINE IN GLANDULAR AND OTHER TISSUES.

By FREDERICK K. SWOBODA.

(*From the Hull Laboratories of Physiological Chemistry and Pharmacology, University of Chicago, Chicago.*)

(Received for publication, May 13, 1920.)

INTRODUCTION.

The work of Williams[1] demonstrates that a substance of unknown structure, a constituent of yeast, is necessary in addition to the ordinary nutrients for the nutrition of yeast cells. This substance Williams concludes, based upon identical occurrence and various properties, is identical with the beri-beri-preventing vitamine. He further found that the growth of single cells of yeast may be used as a simple biological test for this vitamine. His results at the time of the publication of his paper also indicated that this test might be of quantitative value.

The work here presented shows that this vitamine or, as McCollum calls it, the water-soluble B growth-promoting substance can be quantitatively determined by this simple biological method.

The Method.

The Preparation and Extraction of the Tissues.

The tissues examined in the following experiments were obtained as fresh as possible and were immediately treated as follows. The connective tissue and fat were first removed in order to obtain the desired tissue as pure as possible. The tissue was then finely divided, well mixed, and a weighed portion then dried at 3 mm. pressure in a partial ether atmosphere over concentrated H_2SO_4 for 24 hours. The approximate dry weight was then taken. The tissues at this stage were in such a condition that they could be ground up easily with plaster of Paris. This mixture was allowed to stand in a vacuum desiccator for 2 days, reground, and again reground immediately before the extraction. In the cases where not all the tissue was necessary in one experiment and analysis, the total weight of the mixture was obtained and a definite amount of the well mixed material taken for analysis. This was transferred quantitatively into the cup of the Koch extractor which had been thoroughly dried in an oven at 103°C.; the open end of the condenser was connected by means of a cork stopper, covered with tin-foil, with a filled calcium chloride tube which had been bent in order to avoid the possible falling of particles of $CaCl_2$ into the extractor. The tissue was then extracted for 6 hours with commercial absolute ether which had been further dried by standing over metallic sodium for at least 12 hours before use. After most of the ether vapor had been removed from the extractor the extraction with 95 per cent redistilled alcohol was started and continued for 18 consecutive hours. In the preliminary experiments a second alcoholic extraction was made. The alcoholic extract was slowly evaporated to dryness on a steam bath avoiding overheating. The residue was dried in a desiccator over concentrated H_2SO_4, then dissolved in distilled water, and made up to definite volume. Undissolved particles were filtered off after the solution of all vitamine had been insured by slightly warming. This solution was then analyzed by the yeast cell method for its content of alcohol-water-soluble vitamine.

---

[1] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

The Yeast Cell Method.

Preparations.—The yeast cell method has been simplified to a great extent by Williams and myself since the first publication of the method. We found that more uniform results could be obtained by using commercial compressed yeast instead of pure cultures. In all the experiments Fleischmann's compressed yeast was used.

A growth medium containing all the essential inorganic constituents, sugar, and organic nitrogen was prepared as follows:

20	gm.	saccharose (Kahlbaum's).
3	"	$(NH_4)_2SO_4$ (Merck's).
2	"	KH_2PO_4 "
1.5	"	asparagine (Kahlbaum's, twice recrystallized).
0.25	"	$CaCl_2$ (Merck's).
0.25	"	$MgSO_4$ "

These constituents were dissolved in 1 liter of redistilled water. The medium was kept in the ice box after it had been sterilized to avoid any bacterial contamination.

The greatest difficulty was experienced in preparing the cover-slips so that they were perfectly clean, and at the same time that uniform hanging drops could be obtained. The following method was finally adopted as giving the most satisfactory results. After the cover-slips had been thoroughly cleaned, first with soap and water, then with cleaning solution, and rinsed with distilled water until all traces of the cleaning mixture had been removed, they were placed singly on a drying rack especially prepared for this purpose and dried in the oven at 103°C. They were then immersed in a solution containing 1 drop of molten vaseline in 200 cc. of redistilled benzene. The cover-slips were then dried with a towel and dried in the oven at 103°C. It was found that the cover-slips so prepared were very satisfactory, for one can easily obtain drops of uniform size on such a surface, whereas, when they are kept in alcohol, considerable difficulty is experienced in this respect.

The solutions to be tested were prepared as follows. 25 cc. of the sterile medium were measured by means of a pipette into a 150 cc. Erlenmeyer flask, to this was added a known amount of the unknown solution which had been prepared as described above, and then sufficient water to bring the total to 30 cc. Of each concentration duplicates were prepared. A blank test accompanied each set of analyses. All the solutions so prepared and those which had to be preserved for future use, as well as the pipettes and distilled water which were used for the analysis, were now sterilized for 10 minutes at 10 pounds pressure.

The Method.—The following procedure was carried on inside a sterile cupboard prepared for the purpose. This cupboard was sterilized by means of a spray of 50 per cent alcohol 24 hours before use. The sliding glass door was opened only far enough to permit free movement of the hands of the operator.

A cake of Fleischmann's yeast was broken in half inside the sterile cupboard and a piece as large as three pin heads was broken off from the inside of the broken surface by means of a sterile pen and transferred to a test-tube containing about 15 cc. of sterile distilled water. The right concentration of suspension of yeast cells was usually obtained when the solution showed just slight turbidity. A 1 cc. sterile pipette was introduced into the suspension. At the mouthpiece of the pipette a rubber tubing was attached with a good sized cotton plug at one end. By blowing through the tube and pipette a uniform suspension of yeast was insured. A pinch-cock regulated the amount of air needed for suction or pressure. After all the yeast had been suspended, or nearly so, the suspension was allowed to stand for 1 or 2 minutes to allow agglutinated masses of yeast cells or heavier particles to sink to the bottom. 1 cc. of the suspension was then pipetted from the supernatant liquid and transferred to a test solution. By test solution is meant a solution of the same volume as the solution of the water-dissolved alcohol extract to be tested. It is necessary to carry out this preliminary trial, because the concentration of yeast cells in the suspension is of utmost importance. After gently shaking this very dilute suspension for about 1 minute, a sterile pen was dipped into it and 36 drops were made on a cover-slip prepared as described above and immediately inverted on a hollow ground slide prepared with vaseline so as to seal the chamber air-tight. After the time of seeding of the solution had been recorded the drops were immediately examined with the low power of the microscope. A mechanical stage was found to assist greatly in the analysis of the drops. Only those drops were taken into consideration which contained only one or at most two single cells and no foreign contamination. When too many single cells were found per drop on the average, the suspension was diluted with distilled water until satisfactory. On the other hand if too few single cells were found a higher concentration could be obtained by pipetting from nearer the bottom of the first suspension instead of from the surface as indicated above, where a desirable concentration of yeast cells is assumed in the first more concentrated suspension. After the right way of seeding had been determined all the solutions to be tested were treated in the same way. Of each concentration of vitamine to be tested two analyses were made, and for each analysis 36 hanging drops were made and observed, making a total of 72 hanging drops for each concentration. By using a standard pen and cover-slips as described above uniformly sized drops were easily obtained. Every drop was examined microscopically and a record kept for every slide as follows.

Reading for Cover-Slips.

Solution 1a, Table I.

First reading.						6 hr. reading.						18 hr. reading.					
S		2S		2S		1		1 3		2 2		28		14 37	30 24		
	S		S				2		3				34		18		
S					S	3					+	22					
	2S		S	2S			2 3		2	3 2			26 35		41	40 28	
		2S		S				4 2		1				30 32		0	
S			2S		2S	1			2 3		1 1	X			X		26 27

The large square represents the cover-slip.
Every small square represents a hanging drop.
S = one single cell.
2S = two " cells.
+ = more colonies found than could be accounted for.
× = wild yeast colonies.
0 = cell died and autolyzed.

On the average about 65 single cells were taken into consideration for every concentration examined. After the drops had been examined the slide was incubated at 30°C. After 5 to 6 hours incubation the slides were reexamined. Only slight growth had taken place during this time. All the drops which were noted to contain single cells were carefully reexamined and the growth was recorded. Any drop in which more colonies were found than could be expected from the number of single cells recorded in the first reading was excluded from the final consideration. After a total incubation of 18 hours all drops were carefully reexamined and all changes noted. The cells were counted carefully by using the high power of the microscope and recorded. Wild yeast colonies could easily be differentiated at this stage. Drops containing such wild yeast colonies, or cells which had not multiplied at all, or cells which had autolyzed were not considered. All other cells were considered in the final calculation. The minimum and maximum growths are here recorded, but the average growth given is the average of all the readings considered. From the average growth the final values were calculated; *viz.*, the number of cells which would have been obtained if in 18 hours we had used the extract of 1 gm. of tissue for one cell, in the same volume, assuming that the growth would have been proportional, which is, of course, not exactly warranted.

EXPERIMENTAL.

Preliminary Experiments.

Two ripe Brazil nuts were ground up and prepared as described above. Two separate extractions were made of the same sample. The extracts were analyzed separately by the yeast multiplication method. The results obtained are shown in Tables I and II.

For the first analysis relatively smaller quantities were used and accordingly the cell multiplication was smaller than in the subsequent analysis. On the other hand we observed that the values finally calculated are relatively higher for the first analysis. Two factors may account for this. We obtained for the blank test a multiplication of about three and one-half cells on the average from one single cell. Theoretically no multiplication should take place. We know that yeast needs this substance for its development and contains relatively large quantities. In making the yeast suspension, theoretically we suspend only yeast cells, but we undoubtedly dissolve some of the adhering material as well as some dead disintegrated yeast cells, both of which may yield traces of vitamine. A second factor to which we may ascribe this multiplication is the probable storage of vitamine in the yeast cell. The yeast cell when grown on a rich medium will undoubtedly have stored an excess of this particular vitamine. This excess may then consequently be used when this vitamine is absent from the diet. We must consequently take these factors into consideration when a relatively small multiplication of yeast cells takes place.

TABLE I.

Brazil Nut. Analysis I.

No. of solution.*	Minimum growth.	Maximum growth.	Average growth.		No. of cells equivalent to 1 gm. tissue.			
			1	2	Fresh.	Dried.	Fresh.	Dried.
1a	4	18	10.4					
			$_{19}$†	10.4	1,040	1,093		
1b	6	21	10.4					
			$_{35}$	$_{54}$	$_{54}$	$_{54}$	988	1,041
2a	8	24	15					
			$_{10}$	14.6	872	924		
2b	6	25	14.3					
			$_{14}$	$_{24}$	$_{24}$	$_{24}$	$_{78}$	$_{78}$

* Solutions 1a and 1b represent 0.01000 gm. fresh tissue, 0.00948 gm. dried tissue, respectively.

Solutions 2a and 2b represent 0.01675 gm. fresh tissue, 0.01580 gm. dried tissue, respectively.

† The small index numbers indicate the numbers of single yeast cells observed.

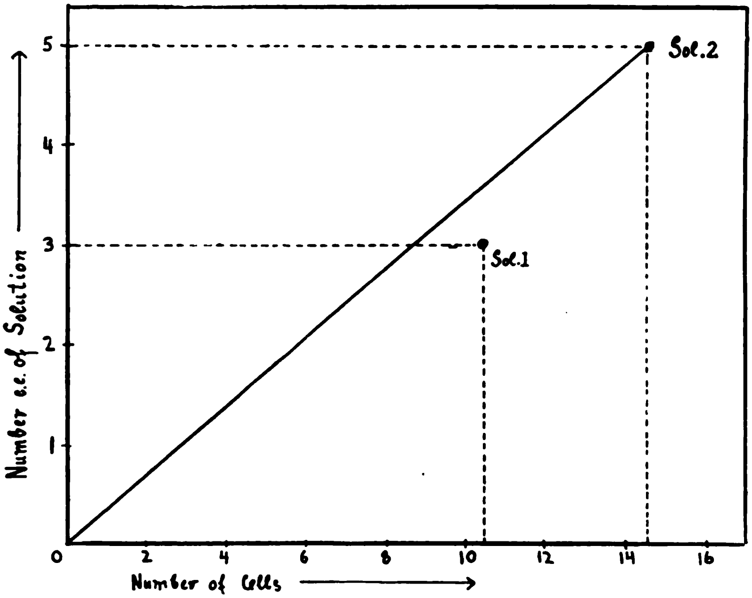
TABLE II.

Brazil Nut. Analysis II.

No. of solution.*	Minimum growth.	Maximum growth.	Average growth.		No. of cells equivalent to 1 gm. tissue.			
			1	2	Fresh.	Dried.	Fresh.	Dried.
1a	11	33	28.5					
			$_{16}$	29.9	885	935		
1b	6	40	30.4					
			$_{15}$	$_{31}$	$_{31}$	$_{31}$	878	927
2a	20	60	48					
			$_{17}$	49	870	918		
2b	16	58	50					
			$_{19}$	$_{36}$	$_{36}$	$_{36}$	$_{67}$	$_{67}$

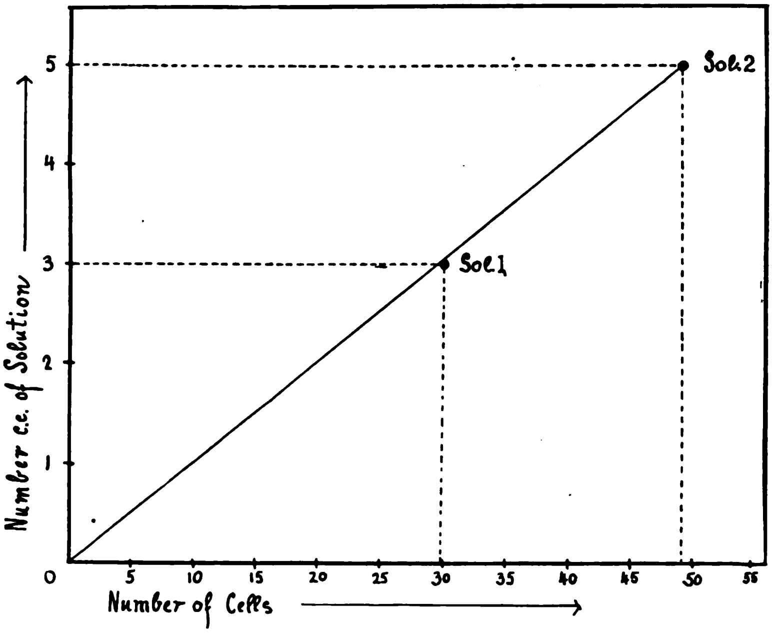
* Solutions 1a and 1b represent 0.03384 gm. fresh tissue, 0.03204 gm. dried tissue, respectively.

Solutions 2a and 2b represent 0.05640 gm. fresh tissue, 0.05340 gm. dried tissue, respectively.



5
4
3
2
1
0
2
4
6
8
10
12
14
16
Sol.2
Sol.1
Number c.c. of Solution
Number of Cells

FIG. 1.



5
4
3
2
1
0
5
10
15
20
25
30
35
40
45
50
55
Sol.2
Sol.1
Number c.c. of Solution
Number of Cells

FIG. 2.

Fig. 1 corresponds to Table I and Fig. 2 to Table II. From these figures we can conclude that these factors are negligible when, under standard conditions, we have ten or more cells developed from one single yeast cell. We find that the value obtained for Solution 1a of Analysis I is theoretically too high, whereas Solution 2 of the same analysis corresponds to the values obtained from the two dilutions of the second analysis.

A second alcohol extraction was made on the residues of both analyses. Vitamine was absent in both cases in these second extractions. Consequently it was considered sufficient to extract the preparation for 14 hours especially since smaller amounts were employed in the succeeding experiments, these being relatively high in vitamine.

To see whether any vitamine was lost during the ether extraction, analyses were made on the aqueous suspensions of the residues of the ether extracts. It was found that no multiplication of yeast cells took place. The extraction with absolute ether, therefore, in confirmation of Williams' findings does not remove any of the specific substance necessary for the multiplication of the single yeast cell. It may be noted from experiments not reported in detail in this paper that when ordinary ether was used in the extraction appreciable vitamine was found to be present in the ether extract. This probably accounts for the varying results obtained by other observers on ether extracts in animal feeding experiments.

The experiments reported in Tables III and IV give further proof for the quantitative value of the results obtained in this first experiment.

As in the first experiment, duplicate analyses of two different dilutions were made and in summary Tables III and IV the calculated vitamine numbers for these different dilutions are reported.

Discussion of Experiments Made on Organs of Internal Secretion.

Various observers have found that glandular organs are relatively high in vitamines. McCollum[2] reports a high vitamine content (both the fat-soluble A and water-soluble B growth-promoting substances) in glandular organs. Funk and Douglas[3] found on the other hand that practically all organs of internal secretion, with a very few exceptions, were atrophied in avian polyneuritis. It was thought, in consequence of these reports, that the substance which promotes the multiplication of the yeast cell and which has been shown to be identical with the beri-beri curative vitamine by Williams[1] must be of great impor-

---

[2] McCollum, E. V., The newer knowledge of nutrition, New York, 1919.

TABLE III.

Summary of the Results of the Vitamine Determinations of Organs of Internal Secretion.

Substance.	Water.	Dilution 1.			Dilution 2.		
		Vitamine number.*		No. of single cells observed.	Vitamine number.		No. of single cells observed.
		Fresh tissue.	Dried tissue.		Fresh tissue.	Dried tissue.	
	per cent						
Liver, Ox 1	66	832	2,448	36	958	2,895	32
“ “ 2	66	986	2,908	49	1,058	3,116	23
“ Dog 1	65.4	405	1,298	37	390	1,247	24
Pituitary, Dog 1	71.8	1,760 (?)	6,250 (?)	36	1,293 (?)	4,600 (?)	28
“ cow	78	411	1,846	29	402	1,802	16
Pineal, “	81	580	3,000	20	576	2,980	19
Kidney, Dog 1	75	354	1,413	52	386	1,546	39
Suprarenal, “ 1	51.3	442	913	36	458	940	30
Testis, “ 1	83.2	155	924	31	116	695	17
“ ram	86	465	3,241	25	450	3,142	13
Ovaries, Dog 2	72.3	247	890	14	264	953	26
“ pregnant dog	74.5	233	915	36	234	917	25
Thyroid, Dog 1	74.6	376	1,360	39	†		
Thymus. “ 2	77	105	455	29	117	507	17
Lymph, “ 1	56	90.7	207	19	74.5	170	31
Pancreas, “ 1	71.8	53	170	28	41	128	20
“ “ 2	68.7	61	216	20	56.5	200	21
Sciatic nerve, Dog 1	50.4	408	820	30	294	590	31

* The vitamine number is the number of yeast cells which would grow from one single cell under the standard condition in 18 hrs. in presence of vitamine extracted from 1 gm. of tissue.

† See discussion under "Thyroid."

---

[3] Funk, C., and Douglas, M., *J. Physiol.*, 1913–14, xlvii, 475.

tance to the organs of internal secretion and that a quantitative study as to its distribution in various organs might throw some light on its function or origin.

In Tables III and IV the summaries of the results of these experiments are reported. Most of the tissues were obtained from dogs. Those tissues marked "Dog 1" were obtained from a young dog which was killed and bled specially for the purpose. Only a few tissues were obtained from other animals as will be indicated in the proper place.

Liver.

In avian polyneuritis Funk and Douglas[3] found the liver in all cases to show a distinct cloudy swelling and in places to have broken cells leaving in some cases only the outline of the interlobular capillaries

McCollum[2] reports that the liver is especially high in content of the water-soluble B growth-promoting substance.

Two different analyses were made of the liver of the ox. From Table III we can see that the liver contains comparatively large quantities of the specific vitamine.

It is evident from Funk's findings that the absence of the beri-beri curative vitamine is fatal to the hepatic cell. He reports further that the fatty droplets, which normally are found in the cells of the liver, are not to be seen in the liver of birds which had shown primary conditions of beri-beri. This fact may indicate that the vitamine is not only necessary for the maintenance of the hepatic cell, but also, that the liver stores the excess of the vitamine.

The liver of Dog 1 also was analyzed. Relatively large quantities of vitamine were found in comparison with other organs of the dog. The vitamine number obtained for the dog liver is relatively much lower than that obtained for the ox liver. Whether this is a constant difference for these animals or whether it varies among individuals of the same species remains to be determined. On the other hand it is well known that the composition of the liver varies considerably from time to time depending on many factors.

Pituitary.

The pituitary obtained from Dog 1 proved to be very small in size and weight. Nevertheless an analysis was made. The calculated vitamine number indicated a high vitamine content. The number is undoubtedly too high, for reasons explained previously.

In order to obtain more convincing results the pituitary of a larger animal was chosen. We were able to obtain the pituitaries of cows directly from the slaughter houses due to the courtesy of Armour and Company. Two whole glands were used for the analyses. A high vitamine content was found. This is in agreement with the findings of Funk and Douglas[3] who report in avian polyneuritis a distinct degeneration of the pituitary.

TABLE IV.

Summary of the Results of the Vitamine Determinations of the Different Parts of the Pituitary of the Cow.

Substance.	Water.	Dilution 1.			Dilution 2.		
		Vitamine number.		No. of single cells observed.	Vitamine number.		No. of single cells observed.
		Fresh tissue.	Dried tissue.		Fresh tissue.	Dried tissue.	
	per cent						
Pituitary, whole............	78	411	1,846	29	402	1,802	16
Anterior lobe, Cow 1......	74.5	384	1,512	39	427	1,684	43
" " " 2......	76.5	377	1,605	40	412	1,750	20
Pars intermedia, Cow 1....	74.7	658	2,660	35	550	2,170	44
" " " 2....	75.5	375	1,740	10	474	1,935	27
Posterior lobe, " 1....	79.6	80.2	392	34	62.2	340	20
" " " 2....	78	104	424	39	115	467	24
Colloid....................	52	183	383	26	172.5	364	58

The distinct physiological function of the three different parts of pituitary, the anterior lobe, pars intermedia, and posterior lobe, is generally admitted today. It was thought of interest to find the location of the vitamine in the pituitary. Two different lots of pituitaries were carefully dissected into the three parts. Analyses were made of the different parts and lots. The summary of the results is reported in Table IV.

A. Anterior Lobe of the Pituitary.—In both analyses a high vitamine content was found. The vitamine number of both analyses is of the same order, the difference being well within the limits of experimental error of this biological test.

The importance of the anterior lobe to growth in general and sex development particularly has been shown by numerous workers. From the recent article of Goetsch[4] we can see that the overactivity of the anterior lobe is always associated with overactivity of the sex glands whereas deficiency is followed by underdevelopment of the sex glands in the young and sexual impotence in the old. This relation is also suggested by the high vitamine content of both the anterior lobe of the pituitary and the testicles and ovaries, as will be shown later.

It is also of interest to learn from Funk and Douglas'[3] paper that in avian polyneuritis especially the anterior lobe showed distinct disintegration, the number of cells being greatly diminished. On the other hand Dutcher[5] reports that tethelin, the extract of the anterior lobe, relieved pigeons of the paralytic symptoms brought about by experimental polyneuritis on a deficient vitamine diet. These facts and the finding of large quantities of vitamine in the anterior lobe give further proof that the vitamine which increases the speed of multiplication of the yeast cell is identical with the beri-beri curative substance as shown by Williams.[1]

B. Pars Intermedia of the Pituitary.—Analyses of two different lots were made. The vitamine numbers obtained for these different lots are both relatively high but differ decidedly from one another. This probably can be explained by the difficulty of separating the posterior lobe from the pars intermedia, the separation never being complete, although the greatest care was employed in the dissection.

Histologically the pars intermedia differs from the anterior lobe in being less vascular. On the other hand, the pars intermedia is well marked off from the posterior lobe which is also often called pars nervosa. It will be seen from the following experiments that the posterior lobe contains relatively little of the vitamine.

---

[4] Goetsch, E., *Surg., Gynec. and Obst.*, 1917, xxv, 229.

[5] Dutcher, R. A., *J. Biol. Chem.*, 1919, xxxix, 63.

C. Posterior Lobe of the Pituitary.—In both analyses the vitamine content was found to be relatively low. As this lobe consists mainly of nervous tissue and shows no change in avian polyneuritis, the low values found are not surprising.

D. Colloid of the Pituitary.—A few hardened pieces of colloid were found during dissection of the glands. The results of the analysis are reported in Table IV.

Only small amounts of vitamine were found. These low vitamine numbers do not necessarily indicate that the colloid is not to be considered the secretion of the gland. These colloid particles which we were able to collect are probably disintegrated material, and may differ chemically very much from the original fluid colloid found in the pars intermedia.

When we calculate the total vitamine content of the gland on the basis of the data of the various parts we obtain numbers which differ only slightly from the vitamine number obtained by analysis of the whole gland.

Analysis I.

	Weight.	Cells per gm. of tissue.	Calculated number of cells for whole.
	gm.		
Posterior lobe	7.169	73	524
Pars intermedia	8.662	598	5,190
Anterior lobe	14.860	407	6,035
Colloid	0.351	176	62
Total glands	31.042	380	11,811
Analysis II.			
Pars intermedia	2.233	444	990
Posterior lobe	0.932	112	104
Anterior "	10.734	389	4,175
Total glands	13.899	379	5,269

The pituitary analyzed as a whole gave a vitamine number of 408 cells per gm. of tissue.

Pineal Gland.

Six pineal glands of cows were obtained directly from the slaughter house. The results of the analysis of these glands are reported in Table III. The vitamine number was found to be somewhat higher than for the pituitary.

The present day knowledge of this gland seems to indicate a close relation of the pineal gland to the sex glands. McCord in his recent paper[6] states in his summary that disturbances of the function of the pineal gland are followed by an early sexual development evidenced in the enlarged genitalia, early change in voice, by precocious mental development, and by general overgrowth of the body. The administration of the pineal gland to young animals is reported to hasten sexual maturity and growth.

Kidney.

Funk and Douglas[3] report in all cases of avian polyneuritis tubular changes, consisting of the swelling and breaking up of the protoplasm of the cells, while the nucleus showed signs of pycnosis. They also report changes of the glomeruli and of the intertubular capillaries and slight edematous conditions of the interstitial tissue. Cooper[7] on the other hand finds that the kidney tissue is a very efficient beri-beri curative.

In agreement with these findings are the results of the vitamine analysis of the kidney of Dog 1. Remarkable quantities of vitamine were found.

Suprarenal.

The suprarenal was found to be slightly changed in avian polyneuritis by Funk. He reports slight degeneration in both the cortical and medullary cells.

The suprarenal gland obtained from Dog 1 was analyzed for vitamine. Considerable quantities were found when the fresh tissue weight is considered.

The relation of the suprarenal to the sex glands was reported in 1836 by Nagel[8] who found that animals with large sex glands and intensely developed sexual activity possess large suprarenals. Vincent[9] states that there is considerable amount of clinical evidence that tumors of the suprarenal cortex are frequently associated with sex abnormalities. Bullock and Sequeira[10] and Glynn[11] report that children with hypernephroma show premature development of the whole body in general, but especially premature development of the sex glands.

---

[6] McCord, C. P., *Surg., Gynec. and Obst.*, 1917, xxv, 250.

[7] Cooper, E. A., *Biochem. J.*, 1914, viii, 250.

[8] Nagel, W., *Arch. Anat., Physiol. u. wissench. Med.*, 1836, 366.

Thyroid.

Funk finds in avian polyneuritis marked degeneration of the cells of the thyroid. Consequently an analysis for vitamine was made.

It was found that when 5 cc. of the water-dissolved extract were used for the analysis most of the yeast cells died during the experiment and the remaining did not grow nearly so well as those of the blank solution. When 3 cc. of the extract were used the growth was equal to that of the blank. Some cells were found to have died during the experiment but not nearly so many as when 5 cc. of the extract were used. When only 1 cc. of the extract was analyzed by the yeast cell method all cells grew and a noticeable increase in the multiplication was noted over the blank test. It is evident that the thyroid extract is poisonous to the yeast cell when added to the medium in large quantities, but when only a small amount is added a noticeable effect on the multiplication of the yeast cells takes place showing the presence of considerable quantities of vitamine.

The relation of the thyroid to other organs of internal secretion has been shown by numerous workers. Hofmeister[12] found that extirpation of the thyroid causes enlargement of the anterior lobe of the pituitary and degeneration of the uriniferous tubules of the kidney. Von Eiselberg[13] finds that after extirpation the sexual glands are affected and growth is retarded. Biedl[14] reports a hypertrophy of the suprarenal. Alquier and Theuveny[15] find after thyroidectomy degenerated testicles and diminished spermatogenesis.

---

[9] Vincent, S., *Surg., Gynec. and Obst.*, 1917, xxv, 294.

[10] Bullock, W., and Sequeira, J., *Tr. Path. Soc. London*, 1905, lvi, 189.

[11] Glynn, E. E., *Quart. J. Med.*, 1911–12, v, 157.

[12] Hofmeister, F., *Beitr. klin. Chir.*, 1894, xii, 245; *Deutsch. med. Woch.*, 1896, xxii, 354.

[13] von Eiselberg, A., *Arch. klin. Chir.*, 1895, xlix, 207. *Deutsch. Z. Chir.*, 1901, lxiv, 259.

Thymus.

In avian polyneuritis the complete disappearance of the thymus is reported by Funk and Douglas.[3] On the other hand we find from the recent work of Uhlenhuth[16] that the thymus as such does not contain the specific growth-promoting substance.

An analysis was made on the thymus of a young dog. Only relatively little vitamine was found. It is interesting to note from a recent article of Hewer[17] that large quantities of thymus are necessary to have an appreciable effect in feeding experiments.

Many divergent statements are found in the literature as to the relation of the thymus to the sex glands. As to the influence of thymectomy Paton[18] observes after removal of the thymus an increase in weight of the testis whereas the results of the experiments of Halnan and Marshall[19] show the opposite; namely, decrease in weight of the testis. On the other hand we find from the work of Pappenheimer[20] that thymectomy had no influence on the development of the young animal. From these results it seems that the thymus has only a secondary function in development and growth.

Testis.

Funk found the testicles greatly atrophied in avian beri-beri. Knowing of the close relation of the organs of internal secretion to the sex glands and particularly knowing that all those organs of internal secretion which are of physiological significance to the sex glands have a high vitamine content, we were not surprised to find large quantities of vitamine in the sex glands.

---

[14] Biedl, A., *Wien. klin. Woch.*, 1901, xiv, 1278; *Wien. Klin.*, 1903, xxix, 281.

[15] Alquier, L., and Theuveny, L., *Compt. rend. Soc. biol.*, 1908, lxiv, 663.

[16] Uhlenhuth, E., *Endocrinology*, 1919, iii, 285.

[17] Hewer, E. E., *J. Physiol.*, 1913–14, xlvii, 479.

[18] Paton, D. N., *J. Physiol.*, 1911, xlii, 267.

[19] Halnan, E. T., and Marshall, F. H. A., *Proc. Roy. Soc. London, Series B*, 1914, lxxxviii, 68.

[20] Pappenheimer, A. M., *Surg., Gynec. and Obst.*, 1917, xxv, 276.

The testicles of two different species were analyzed. The testicles of Dog 1, which was a young dog and probably not full grown, showed a high vitamine content. The testicles of a ram also were analyzed and were found to be very much higher in vitamine. Table III gives the results of these experiments.

An attempt was made to dissect out of the testicle of the ram the interstitial glands. Only a few mg. of fresh material were obtained. When analyzed by the yeast cell method only a slight increase of the speed of multiplication was noted over that of the blank solution. As only very little material was to be accounted for, even a very little increase in growth indicates a high vitamine content of the interstitial glands. These experiments will be repeated on a vasectomized animal and will be reported later.

Ovaries.

In avian polyneuritis Funk found the size of the ovaries greatly diminished.

Two fully developed female dogs were killed. One was pregnant and the other not. The ovaries were removed and analyzed for vitamine. In the pregnant dog the ovaries were twice as heavy as those obtained from the normal dog and showed the presence of many corpora lutea. The vitamine concentration is of the same order in both cases, but since the ovaries were twice as heavy in the pregnant as in the non-pregnant animal the total amount is about twice as much vitamine. Whether this is related to the corpora lutea content has not been determined.

Sciatic Nerve.

Disintegration of the axis cylinder of the sciatic nerve has frequently been reported as reason for the paralytic symptoms in beri-beri. The sciatic nerve was obtained from Dog 1 and analyzed for vitamine. Considerable amounts of vitamine were found in proportion to the fresh weight of the nerve.

Lymph Gland.

The lymph gland of Dog 1 was analyzed for vitamine. The results of the analysis are reported in Table III.

Only traces of vitamine were found.

Pancreas.

Funk and Douglas[3] report that in avian polyneuritis the pancreas was not diminished in size nor histologically changed.

The pancreas of Dog 1 when analyzed gave an exceedingly low vitamine number.

The finding is partly contradictory to the finding of Williams[1] who found that pancreatin decidedly increased the speed of multiplication of the yeast cell, and to the findings of Eddy[21] who reported that the feeding of the pancreas increases the growth of white rats when added to a diet deficient in water-soluble B growth-promoting substance. Eddy in his feeding experiments used an evaporated extract obtained by extracting a sheep pancreas with 95 per cent alcohol of 0.8 per cent HCl strength. Hydrolysis will certainly take place to a certain limit in such an acid concentration. Previous observers also found that hydrolyzed extracts are more curative for beri-beri than unhydrolyzed extracts. It is very likely that the pancreatin used by Williams was also slightly hydrolyzed.

These results would indicate that we might possibly have a vitamine precursor present in the pancreas. In order to verify the results obtained from the analysis of the first pancreas another one was obtained. The second pancreas was obtained from the same normal female dog from which the ovaries were obtained. The results are identical with the first determination and verify the vitamine number obtained for the fresh pancreas.

DISCUSSION.

From the foregoing experiments it is clear that the yeast cells, when grown on a synthetic medium containing sugar, asparagine, ammonium nitrogen, potassium, calcium, magnesium, sulfate, and phosphate, are dependent for their growth on a substance, soluble in 95 per cent alcohol and water, but of unknown chemical structure. This substance stimulates the growth of the yeast cell, or the multiplication of the cell, so that a unit weight of this substance is essential for the growth of a definite number of yeast cells. This finding was made the basis of the present method for the quantitative determination of this substance which in all probability is identical with the beri-beri curative vitamine. In all experiments, except the one with the thyroid extract, the quantitative relation between unit weight of vitamine and number of yeast cells produced has been demonstrated. In the case of the thyroid extract we undoubtedly have to deal with a substance which is toxic to the yeast cells, in addition to the growth-promoting substance. So we have in the thyroid extract really not an exception, but a more complicated situation.

---

[21] Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.

The foregoing experiments also show the distribution of vitamine in the animal body. We find the pituitary and pineal gland highest in vitamine content. Although little may be known at present of the function of these glands still there seems to be enough experimental evidence to indicate their close relation to growth and sex development. Most of the other organs of internal secretion—suprarenal, testis, ovaries, and thyroid, all of importance to growth development—contain about the same amount of vitamine per gram of dried tissue. A somewhat higher concentration of vitamine is found in liver and kidney. On the other hand little was found in those glands which contain much nuclear material—the thymus, pancreas, and lymph gland. This fact may be partly contradictory to the fact that a high amount is found in the yeast cells also containing large quantities of nuclear material. The experiments with the pancreas seem to indicate that the vitamine is present here in the form of vitaminogen. This vitaminogen may be acted upon by acids or possibly ferments to liberate the active growth-promoting substance. It is interesting to note that the sciatic nerve also contains remarkable quantities of vitamine.

The experiments of Funk and Emmett and Allen[22] showed that all these organs were primarily affected by vitamine deficiency. The finding of vitamine in all these organs seems to indicate the importance of vitamine for growth or sex development. Whether this vitamine is a growth-promoting substance, *e.g.* whether it facilitates the building up of the animal body in general only, or whether it is particularly a sex-promoting substance, remains to be determined.

---

[22] Emmett, A. D., and Allen, F. P., *J. Biol. Chem.*, 1920, xli, p. liii.

SUMMARY.

1. The biological test of Williams for the detection of small quantities of the water-soluble B vitamine was developed and found to be of quantitative value.

2. The method was applied and the vitamine content of various organs determined. A summary of all determinations, giving the different vitamine numbers, is found in Tables III and IV.

3. From the results of these determinations it is evident that this specific vitamine is present in large quantities in most of the organs of internal secretion which are of developmental importance, whereas it usually is present in very much lower concentrations in other organs examined. The liver and kidney, however, are high in vitamine content.

4. The tissues high in nuclear material, such as thymus and lymph gland, were found low in vitamine content.

5. The fresh pancreas was found low in activity. It is suggested that high content reported by others may be due to hydrolysis.

6. The thyroid was the only organ in which an increase in concentration of the vitamine fraction caused a toxic action. Without proper dilution the vitamine activity would not be observed.

7. These vitamine determinations brought additional evidence that the substance which increases the multiplication of the yeast cell is identical with the beri-beri curative vitamine.

In conclusion I wish to acknowledge my indebtedness to Professor F. C. Koch and Dr. R. J. Williams for valuable advice given me throughout the research, and Dr. M. Hines for her assistance in the dissection of different organs.

STUDIES OF AUTOLYSIS.

VI. EFFECT OF CERTAIN COLLOIDS UPON AUTOLYSIS.

By H. C. BRADLEY and H. FELSHER.

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.*)

(Received for publication, September 2, 1920.)

In 1907, Ascoli and Izar[1] published the first of a series of interesting observations on the effect of inorganic colloids upon the rate and extent of autolysis. The results reported in this first paper show that the metallic hydrosols, gold, silver, and platinum, are able to increase autolysis very markedly. Thus 9 mg. of colloidal silver in a digest containing 20 gm. of liver were sufficient to cause a 100 per cent increase of digestion in a period of 60 hours. The three metals were found to behave exactly alike, except for minor quantitative differences. The effect appeared to be due, therefore, to the colloidal state rather than to any chemical action which would be specific for the element added.

Autolysis was determined by removal of proteins by heat coagulation and acetic acid, and the estimation of non-coagulable nitrogen in the filtrate. In subsequent papers the coagulation was performed in the presence of an excess of KH_2PO_4. The metallic hydrosols were found to increase autolysis in slightly alkaline mixtures as effectively as in the normally acid digests. This statement is of particular interest when it is recalled that alkaline or even neutral tissue pulps normally do not autolyze at all. The colloids would appear to be remarkably effective if they can overcome the regular inhibitory effect of alkali.

The authors give three possible explanations of the mechanism of increased autolysis; (1) the hydrosols may activate the enzymes, (2) they may counteract some antiautolytic substance in the mixture, or (3) they may have direct catalytic action in themselves facilitating the hydrolysis of liver proteins along with the enzymes. The authors evidently incline to the last hypothesis, and find in it a confirmation of the point of view suggested by Bredig[2] 8 years before. Bredig indicated the similarity in the action of colloidal sols and the enzymes which decompose hydrogen peroxide. It led to the rather widely accepted hypothesis that the enzymes accomplish their catalytic effects by virtue of their colloidal character chiefly, and that therefore other typical digestive reactions besides the decomposition of hydrogen peroxide might be simulated by inorganic colloids.

---

[1] Ascoli, M., and Izar, G., *Berl. klin. Woch.*, 1907, xliv, 96, 659.

The experiments of Ascoli and Izar offer confirmation too, and explanation of the fact already announced by Galeotti and Todde[3] and Robin and Bardet,[4] that injections of metallic hydrosols lead to increased nitrogen elimination as a result of some deep seated increase of catabolism. The impression gained from the paper of Ascoli and Izar and their subsequent publications is that the colloidal sols, acting probably like hydrolytic enzymes themselves, accelerate the hydrolysis of proteins both *in vitro* and *in vivo.* Such increased protein and purine breakdown results in increased total nitrogen elimination and in particular increased uric acid output. The facts developed by Ascoli and Izar in their studies correlate, therefore, very perfectly with the finding of increased catabolism, produced by injecting colloidal sols.

The number of inorganic hydrosols which increase autolysis was found to be very large. Indeed, not one of the series investigated by Ascoli and Izar failed to produce marked acceleration and increase of autolysis, varying from 50 to 500 per cent in some instances. Metallic sols were prepared usually by the Bredig method and included gold, silver, platinum, palladium, copper, etc. Colloidal hydroxides were used, as of iron, aluminum, manganese. Sulfides, as arsenic sulfide, As_2S_3, were very potent in increasing the digestion. The variety used is so wide, including positively and negatively charged colloids, that the conclusion seems inevitable that the effect is attributable in some way to the colloidal state, the only factor in common to all the sols tried. The chemical composition of the particular colloid has little to do with the reaction. The authors do conclude, however, in a later paper,[5] that the intensity of the effect is somewhat dependent upon the group to which the element belongs.

---

[2] Bredig, G., Anorganische Fermente, Leipsic, 1899.

[3] Galeotti, G., and Todde, *Lo Speremental*e, 1902, 341, quoted by Ascoli, M., and Izar, G., *Biochem. Z.*, 1907, v, 394.

[4] Robin and Bardet, *Bull. Therap.*, 1904–05, quoted by Ascoli, M., and Izar, G., *Biochem. Z.*, 1907, v, 394.

Our own study of autolysis, and much of the earlier work, has established the fact that acids are the usual accelerators of this reaction. Indeed so many of the examples of acceleration recorded in the literature have proved to be due to the acidity, rather than to other assigned reasons, that one is inclined to assume that this is the only true cause of acceleration, aside from that resulting from the direct addition of digestible protein to an autolyzing tissue pulp. Thus the accelerations reported by Preti[6] for lead, iron, and manganous salts are found to be due, not to the metallic ion at all, but to the acidity produced when such salts hydrolyze in water. The acceleration due to bromine and iodine, phosphorus under certain conditions, carbon dioxide, salicylic and boric acids, etc. are all associated with increased acidity (Morse,[7] Yoshimoto,[8] Bradley,[9] etc.).

Not only are acidity and added protein the only firmly established conditions for increased autolysis of which we are aware, but from the logic of our own experiments we are forced to the conclusion that these two mechanisms are identical, and that acids are effective agents in the reaction because they increase the mass of available protein substratum in the digest. They convert the great mass of normally undigested and indigestible proteins of the tissue into proteins which are attacked by the enzyme. The effect of adding acid is therefore exactly like that of adding acid albuminate or casein or gelatin; it increases the mass of substratum and so causes increased amino-acid production per unit of time, and a larger absolute amount when equilibrium is attained.

It was recognized early in our study of autolysis that the results of Ascoli and Izar were likely to constitute some sort of an interesting exception to this general behavior of autolyzing gland tissue. No patent explanation of this behavior of colloidal solutions was forthcoming which could relate it in any way with the addition of acid. We have therefore purposely put off the reinvestigation of this problem until we felt more familiar with the normal and usual mechanism of the reaction, and until we were in better possession of the fundamental factors that control it. Feeling sure of the ground already covered we have now proceeded to take up the study of the effect of colloids upon the reaction, with a view to determining whether they affect the enzyme, or the proteins, to produce the increased autolysis. *To our complete surprise we have thus far failed to find any real case of undoubted acceleration of autolysis by the presence of an inorganic colloid.* In view of the many experiments performed by Ascoli and Izar, and the great detail with which they are reported, we do not wish to offer further comment at this time, but merely present our data as obtained thus far. Some accelerations have appeared in our results. The more prolonged and complete the purification of the sol by dialysis, the more certain it is that the sol would be without effect. As a rule further dialysis of the colloid has reduced or removed this acceleration along with small amounts of acids or acid-reacting electrolytes. It is possible that such prolonged dialysis alters the physical character of the colloid and thus renders it inert, though of this we have no visible evidence. The more likely explanation appears to us to be the removal of traces of acids or electrolytes producing acid by hydrolysis. The following protocols will illustrate how completely at variance are our results with those published by Ascoli and Izar.

---

[5] Ascoli, M., and Izar, G., *Biochem. Z.*, 1909, xvii, 361.

[6] Preti, L., *Z. physiol. Chem.*, 1909, lx, 317.

[7] Morse, M., *J. Biol. Chem.*, 1915, xxii, 125.

[8] Yoshimoto, S., *Z. physiol. Chem.*, 1908–09, lviii, 341.

[9] Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113.

EXPERIMENTAL.

The digests were made with liver pulp exactly as in previous papers. Titrations of amino-acids constitute our criterion of autolysis in all cases. In certain experiments we have used in addition the methods of heat coagulation as described by Ascoli and Izar. We believe the trichloroacetic acid method of precipitating the proteins to be more reliable, however, than the methods of coagulation by heat.

Experiment 1. Effect of Colloidal Silver.—Two types of silver sols were used, one prepared by arcing under distilled water with silver electrodes, the other by reducing a silver nitrate solution with phosphorus dissolved in ether. The solutions were deep brown, filtered readily, and were stabilized with small amounts of dialyzed gelatin. All these solutions would decompose hydrogen peroxide.

Silver Sol I.—This was prepared by arcing between silver electrodes under distilled water until the solution was a deep brown. The solution was kept surrounded by cold water during the preparation, but the temperature was frequently as high as 80°C. After filtering the sol a few cc. of 1 per cent gelatin were added to stabilize the colloid. There was not enough available protein added to disturb the results by the digestion of the stabilizer. The results are given in Table I.

TABLE I.

Silver Sol I; Beef Liver.

	Days.				Net gain.
	0	2	5	7	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Control	0.55	1.20	1.30	1.30	0.75
" + 1 cc. Ag Sol I	0.55	1.30	1.30	1.35	0.80
" + 5 " Ag " I	0.55	1.30	1.35	1.35	0.80
" + 10 " Ag " I	0.55	1.30	1.30	1.35	0.80
" + 25 " Ag " I	0.55	1.30	1.30	1.35	0.80

Silver Sol II.—This was prepared in the same way, filtered, stabilized, and dialyzed (Table II).

TABLE II.

Silver Sol II; Beef Liver.

	Days.				Net gain.
	0	2	5	12	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Control	1.10	1.50	1.60	2.10	1.00
" + 10 cc. Ag Sol II	1.10	1.40	1.75	2.30	1.20
" + 25 " Ag " II	1.10	1.40	1.80	2.35	1.25
" + 50 " Ag " II	1.10	1.40	1.70	2.30	1.20

Silver Sol III.—Dialyzed gelatin was added to distilled water to a strength of 0.03 per cent. The sol was prepared by the Bredig method with the gelatin present. The resulting sol was alkaline and showed ammonium compounds and silver salts in solution, from the decomposition of the gelatin. The preparation was dialyzed for many days till neutral and free from salts of silver. This was a very strong sol, appearing black except in thin layers, when it showed the typical brown color. It decomposed hydrogen peroxide rapidly. The results are given in Table III.

TABLE III.

Silver Sol III; Beef Liver.

	Days.				Net gain.
	0	1	3	5	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Control	0.40	0.95	1.10	1.10	0.70
" + 0.5 cc. Ag Sol III	0.40	1.00	1.05	1.10	0.70
" + 2.5 " Ag " III	0.40	1.00	1.05	1.10	0.70
" + 15 " Ag " III	0.45	1.00	1.05	1.10	0.65
" + 30 " Ag " III	0.45	1.00	1.05	1.10	0.65
" + 50 " Ag " III	0.40	1.00	1.05	1.10	0.70

Silver Sol IV.—This was made up from a silver nitrate solution, by the addition of ether saturated with phosphorus. After the reduction was complete, films and particles of metallic silver were filtered off, the sol was stabilized with gelatin, and the mixture dialyzed till free from phosphoric acid. It decomposed hydrogen peroxide less rapidly than No. III, and was evidently less concentrated (Table IV).

TABLE IV.

Silver Sol IV; Beef Liver.

	Days.					Net gain.
	0	1	2	3	10	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Control	0.50	0.90	1.00	1.00	1.10	0.60
" + 1 cc. Ag Sol IV	0.50	0.90	1.00	1.00	1.10	0.60
" + 5 " Ag " IV	0.50	0.90	1.00	1.10	1.10	0.60
" + 10 " Ag " IV	0.50	0.90	1.00	1.00	1.10	0.60
" + 25 " Ag " IV	0.50	0.90	1.00	1.00	1.10	0.60
" + HCl	0.50	1.70	2.40	2.65	3.20	2.70

With these four silver sols then, stabilized as were Ascoli and Izar's, and carefully freed from impurities by prolonged dialysis in collodion bags against distilled water, no results could be detected. They neither accelerated nor inhibited the course of autolysis. Yet silver sols were very effective in the hands of Ascoli and Izar and were used as standards with which to compare other hydrosols. All the sols were thoroughly stable, and after several months showed mere traces of filterable precipitates or none at all.

Experiment 2. Effect of Fe(OH)₃.—Having failed to confirm the results reported by Ascoli and Izar with colloidal silver, we took up another colloid, easy of preparation, stable, and readily purified. $Fe(OH)_3$ may be obtained by pouring $FeCl_3$ of proper strength into distilled water. Such a preparation is always acid from the hydrolysis of the $FeCl_3$. By prolonged dialysis it may, however, be obtained quite neutral. It is obtainable also on the market. Merck's "dialyzed iron," a 5 per cent Fe_2O_3 commercial hydrosol, was used in our experiments first. The preparation gave evident acceleration. It was also quite acid. When, however, it was thoroughly dialyzed for a week against many changes of distilled water, it no longer had any effect on the reaction (Table V).

TABLE V.

Colloidal Ferric Hydroxide; Beef Liver.

	Days.					Net gain.
	0	1	2	3	10	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Control	0.50	0.95	1.05	1.05	1.30	0.80
" + 0.5 cc. $Fe(OH)_3$	0.50	0.95	1.05	1.10	1.30	0.80
" + 2.5 " $Fe(OH)_3$	0.50	0.95	1.05	1.10	1.25	0.75
" +10.0 " $Fe(OH)_3$	0.50	0.95	1.15	1.15	1.35	0.85
" +50.0 " $Fe(OH)_3$	0.50	0.95	1.05	1.20	1.40	0.90
" +25.0 " $Fe(OH)_3$(commercial)	0.50	1.65	2.00	2.40	2.70	2.20

This experiment has been repeated a number of times with the same results in each case. As long as the hydrosol contains chlorides which can be found in the dialysate after a few hours it will accelerate autolysis. When the chloride test becomes quite negative, the accelerating action ceases. In certain of the experiments in which this hydrosol was used, Ascoli and Izar controlled its action by adding to a digest an amount of HCl equal to the acidity of the iron sol. It is inexplicable that their figures show no acceleration of autolysis on this addition of HCl. They do not state how they determined what the acidity of the hydrosol was, so that we have no means of judging the accuracy of their estimate. It is interesting to note that the ferric hydroxide present in these digests does not inhibit the digestion in the least.

This shows clearly that the hydroxide is not considerably dissolved by the organic acids present in the digests, for if it were it would tend to maintain the neutrality of the mixture and so prevent or inhibit autolysis. Small amounts of soluble ferric and ferrous salts are produced, so that in digests carried on for many days or weeks there is sufficient of the iron salts to make the end-points in titration difficult, and so cause the figures to appear somewhat higher than they should be. Thus in the long continued autolysis, the digests containing the hydrosol do show slightly higher figures if titrated in the usual way. If, however, the iron is first separated by the addition of sodium acetate and boiling, the filtrate titrates exactly like the control.

Experiment 3. Effect of Arsenic Sulfide.—This sulfide was found by Ascoli and Izar to be one of the best accelerators of liver autolysis. Hydrosols were prepared by dissolving arsenic oxide in boiling water. The solution was filtered, cooled, and hydrogen sulfide run in till the maximum color developed. This solution was then filtered and stabilized either with gelatin or with starch. Starch was found to be very effective as a stabilizer, preventing the precipitation of the sulfide by acids and electrolytes unless of considerable concentration. The stabilizing action of either gelatin or starch would quickly be eliminated in a liver digest since the enzymes present convert both of these colloids to non-colloidal compounds. They are useful only in preventing precipitation during the process of purification. The sols so prepared were dialyzed for several days, and a stream of air was also blown through them for 8 or 10 hours to remove the excess of hydrogen sulfide. The removal of excess hydrogen sulfide is very important. A small amount of that gas is sufficiently acid to increase autolysis quite markedly (Table VI).

TABLE VI.

Hydrogen Sulfide Gas; Beef Liver.

	Days.			Net gain.
	0	2	8	
	cc.	*cc.*	*cc.*	*cc.*
Control	0.70	2.80	3.50	2.80
" + H_2S (10 sec. bubbling)	0.70	4.00	5.00	4.30
" + H_2S (2 min. ")	0.70	4.00	4.85	4.15
" + H_2S (5 " ")	0.70	4.00	4.80	4.10

We have found considerable accelerations when using sols of arsenic sulfide which had been dialyzed only a few days. The identical sols after more prolonged dialysis still showed some accelerating action. Arsenic ulfide slowly hydrolyzes in water to arsenic trioxide and hydrogen sulfide, so that the effect of the colloid is to add hydrogen sulfide slowly to the mixture (Table VII).

TABLE VII.

Arsenic Sulfide Sol; Beef Liver.

	Days.		Net gain.
	0	4	
	cc.	*cc.*	*cc.*
Control	0.60	2.80	2.20
" + 1 cc. As_2S_3 sol	0.60	3.15	2.55
" + 5 " As_2S_3 "	0.60	3.20	2.60
" + 25 " As_2S_3 "	0.60	3.30	2.70
" + 50 " As_2S_3 "	0.60	4.10	3.50
" + 100 " As_2S_3 "	0.60	5.00	4.40

It may be that the accelerations obtained by Ascoli and Izar were due to the small remaining acidity and to hydrolysis of the sulfide. It is interesting to note that this colloid does not have any apparent effect in decomposing hydrogen peroxide, and is oxidized only very slowly.

SUMMARY.

Following the methods of Ascoli and Izar as closely as possible we have tried the effect of several hydrosols upon the autolysis of the liver. Where accelerations were demonstrated acidity could also be detected, and in preparations highly purified by long dialysis no accelerating action could be found.

The colloids used were silver sols, colloidal ferric hydroxide, and arsenic sulfide, all of them reported as very effective by Ascoli and Izar. We have thus far failed to confirm any of the findings reported by these authors.

EXPERIMENTS ON CARBOHYDRATE METABOLISM AND DIABETES.

III. THE PERMEABILITY OF BLOOD CORPUSCLES TO SUGAR.

By MARY B. WISHART.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, August 9, 1920.)

The subject of the permeability of the blood corpuscles to sugar was reviewed in 1913 by Allen[1] and in 1917 by Gradwohl and Blaivas.[2] Analyses covering both plasma and corpuscle sugar during glucose tolerance tests in human subjects were made by Bailey.[3] A large number of comparative analyses upon diabetic patients have already been published.[4] According to some statements in the literature, the corpuscles sometimes contain little or no sugar; according to others, their sugar content does not differ greatly from that of the plasma; and another belief has been that the corpuscles take up sugar more slowly and retain it longer, so that their sugar content is low in the early stages of hyperglycemia but above that of the plasma in the declining stages. The preponderance of experience is that the sugar content of the corpuscles is usually a little below that of the plasma.

The present investigation was carried out as part of an inquiry into the permeability of body structures for sugar, with a special view to any changes occurring in diabetes. Several other physiological and pathological conditions were considered, also the possible differences between species and between permeability *in vivo* and *in vitro*. Numerous analyses of the corpuscle mass after thorough centrifugation were performed, but these were rejected in favor of calculations based on comparative analyses of whole blood and plasma. The method of analysis was that of Lewis and Benedict.[5] The reasons for the usual lack of strict agreement between the analyzed and calculated corpuscle sugars and for giving preference to the latter are obvious. Attention may be directed also to the necessity of precautions for complete precipitation of protein when dealing with corpuscles or even

---

[1] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 6.

[2] Gradwohl, R. B. H., and Blaivas, A. J., *J. Lab. and Clin. Med.*, 1916–17, ii, 416.

[3] Bailey, C. V., *Arch. Int. Med.*, 1919, xxiii, 455.

[4] Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919.

TABLE I.

Normal Dogs Receiving Glucose by Stomach.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Volume.	Glucose.							
Dog B2-98. Weight 4.3 kg. Given 9 gm. glucose per kg. by stomach tube in 30 per cent solution.								
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.062	0.079	0.026	3.04	72	32	Blood before feeding.
13	Slight.	0.159	0.232	0.017	13.64	70	34	½ hr. after "
5	0.22	0.345	0.371	0.274	1.35	63	27	1¼ hrs. " "
14	Faint.	0.116	0.125	0.086	1.45	45	23	14½ " " "
Dog C3-22. Weight 36 kg. Given 15 gm. glucose per kg. by stomach tube in 54 per cent solution.								
	0	0.089	0.111	0.035	3.17	71	29	Blood before feeding. Temperature 101.4° F.
16	Faint.	0.161	0.222	0.043	5.16	87	34	½ hr. after feeding. Temperature 101.1° F.
13	0.15	0.147	0.159	0.124	1.28	86	35	1 hr. after feeding. Temperature 101.0° F.
39	0.63	0.156	0.175	0.122	1.43	85	36	2½ hrs. after feeding. Temperature 100.6° F.
41	0.48	0.179	0.208	0.127	1.63	110	36	3¼ hrs. after feeding. Temperature 100.5° F.

---

[5] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

whole blood. Traces of unprecipitated protein may have an action upon either copper or picric solutions, and such small slips of technique are probably the commonest explanation of results which show the sugar concentration in the corpuscles as high as or higher than that in the plasma. Even with the greatest care in all particulars, any long series will show occasional discrepancies in the ratio of corpuscle sugar to plasma sugar which probably represent analytical errors.

TABLE II.

Normal Dogs Receiving Glucose Parenterally.

Urine.								
Volume.	Glucose.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
Dog C3-07. Weight 14.3 kg. Given 4 gm. glucose per kg. subcutaneously in 30 per cent solution.								
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.096	0.100	0.087	1.14	64	31	Blood before injection.
164	0	0.094	0.099	0.083	1.19	45	33	18¾ hrs. after injection. Fed regular diet.
118	0	0.133	0.139	0.114	1.21	50	24	26½ hrs. after injection.
1,279	0	0.115	0.177	0.109	1.07	54	26	42 " " "
Dog C 3-36. Weight 17 kg. Given 6 gm. glucose per kg. intraperitoneally and 4 gm. per kg. subcutaneously in 30 per cent solution. Total duration of injections 25 min.								
		0.093	0.115	0.063	1.82	114	43	Blood before injection.
		0.286	1.000	0.160	6.25	148		5 min. after end of injection.
		0.578		0.143*		122		25 min. after end of injection.

* Determined.

Tables I and II show the results at various intervals after enteral and parenteral doses of glucose in normal dogs. The corpuscle sugar is regularly below that of the plasma, both during the rise and during the decline of hyperglycemia; and sometimes, as in Dogs B2-98 C3-22, the difference is extreme.

Tables III to VI likewise illustrate the effect of glucose by stomach or subcutaneously in dogs depancreatized short of diabetes. There are minor differences between individual dogs but no perceptible change of corpuscle permeability due to the operation. Control doses of plain saline also gave no change. This series included Dog B2-01, which was characterized by a noticeably low renal threshold for sugar,[6] but the corpuscles appeared no more permeable than in other dogs.

TABLE III.

Dog B2-60.

After removal of only the splenic process of the pancreas. Weight 45 kg.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Volume.	Glucose.							
4 gm. glucose per kg. by stomach tube in 30 per cent solution.								
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.133	0.136	0.127	1.07	40	34	Blood before feeding.
70	0.76	0.200	0.278	0.042	6.61	52	33	2½ hrs. after "
260	0	0.095	0.105	0.073	1.44	60	32	10½ " " "
35	0	0.088	0.093	0.076	1.22	49	30	23 " " "
4 gm. glucose per kg. subcutaneously in 30 per cent solution.								
	0	0.086	0.081	0.097	0.83	54	31	Blood before injection.
55	Slight.	0.125	0.135	0.113	1.19	96	46	6 hrs. after "
65	0.26	0.103	0.143	0.056	2.55	88	46	23 " " "
600 cc. 0.85 per cent NaCl solution subcutaneously.								
	0	0.105	0.105	0.105	1.00	71	41	Blood before injection.
45	0	0.117	0.117	0.117	1.00	75	35	3 hrs. after "
92	0	0.100	0.105	0.088	1.19	60	30	5½ " " "
312	0	0.091	0.110	0.058	1.89	77	37	24 " " "

---

[6] Allen, F. M., and Wishart, M. B., *J. Biol. Chem.*, 1920, xlii, 420, 439.

TABLE IV.

Dog B2-61.

After removal of ¾ of the pancreas. Weight 6 kg.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
Volume.	Glucose.							
3 gm. glucose per kg. in 30 per cent solurtion by stomach tube.								
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.122	0.143	0.090	1.59	87	40	Blood before feeding.
50	0.32	0.105	0.112	0.103	1.09	79	60	3 hrs. after "
50	0	0.100	0.111	0.080	1.39	80	36	10½ " " "
10	0	0.095	0.011	0.057	1.89	51	30	22 " " "
3 gm. glucose per kg. in 30 per cent solution subcutaneously.								
	0	0.105	0.106	0.104	1.02	110	49	Blood before injection.
7	Slight.	0.118	0.147	0.079	1.86	120	43	3½ hrs. after "
21	Faint.	0.111	0.111	0.111	1.00	95	44	22 " " "
50 cc. 0.85 per cent NaCl solution subcutaneously.								
	0	0.122	0.128	0.110	1.16	63	35	Blood before injection.
25	0	0.105	0.118	0.081	1.45	77	36	3½ hrs. after "
4 gm. glucose per kg. in 30 per cent solution subcutaneously.								
	0	0.105	0.133	0.053	2.51	65	35	Blood before injection.
56	Faint.	0.100	0.109	0.080	1.36	91	34	18 hrs. after " Fed regular diet.
35	0	0.161	0.167	0.147	1.13	65	30	26 hrs. after injection.
175	0	0.114	0.117	0.109	1.07	76	39	42 " " "

TABLE V.

Dog B2-00.

Weight 14 kg. Depancreatized not quite to point of diabetes.

3 gm. glucose per kg. in 30 per cent solution by stomach tube.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.122	0.122	0.122	1.00	52	36	Blood before feeding.
0	0.161	0.238	0.115	2.07	58	34	1 hr. after “
0	0.100	0.110	0.080	1.37	65	34	8 hrs. “ “
0	0.096	0.094	0.100	0.94	61	29	10 “ “ “
0	0.091	0.091	0.091	1.00	58	28	15 “ “ “
0	0.078	0.110	0.028	3.93	71	39	24 “ “ “
0	0.105	0.111	0.094	1.18	58	35	27 “ “ “
0	0.113	0.118	0.102	1.15	60	32	29½ “ “ “
0	0.102	0.108	0.089	1.21	51	32	32 “ “ “

3 gm. glucose per kg. in 30 per cent solution subcutaneously.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
Volume.	Glucose.							
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.065	0.065	0.065	1.00	100	40	Blood before injection.
	Slight.	0.134	0.163	0.103	1.58	105	49	2¾ hrs. after “
	0.69	0.134	0.154	0.115	1.34	120	52	8 “ “ “
	0.82	0.098	0.109	0.086	1.26	100	49	11 “ “ “
	Faint.	0.100	0.118	0.075	1.57	97	42	23½ “ “ “

140 cc. 0.85 per cent NaCl solution subcutaneously.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.114	0.116	0.110	1.05	59	34	Before injection.
Faint.	0.105	0.114	0.085	1.34	58	32	1 hr. after injection.
0	0.100	0.130	0.036	3.61	67	32	3½ hrs. “ “
0	0.128	0.130	0.122	1.06	40	27	6½ “ “ “
0	0.112	0.118	0.097	1.21	58	29	10 “ “ “
0	0.100	0.102	0.095	1.07	57	29	23½ “ “ “

TABLE VI.

Dog B2-01.

Weight 14 kg. Depancreatized not quite to point of diabetes.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
4 gm. glucose per kg. in 30 per cent solution by stomach tube.							
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.090	0.116	0.068	1.70	109		Blood before feeding.
Faint.	0.179	0.192	0.164	1.17	114	47	1 hr. after “
	0.100	0.106	0.092	1.15	100	45	3 hrs. “ “
0	0.105	0.131	0.079	1.65	104	50	7 “ “ “
0	0.089	0.132	0.029	4.55	101	42	24 “ “ “
4 gm. glucose per kg. in 30 per cent solution subcutaneously.							
	0.099	0.172	0.034	5.06	105	53	Blood before injection.
	0.166	0.244	0.096	2.54	124	53	½ hr. after “
	0.192	0.266	0.115	2.31	104	49	2 hrs. “ “
	0.217	0.284	0.160	1.77	107	54	5½ “ “ “
	0.200	0.324	0.080	4.05	106	51	7 “ “ “
	0.166	0.230	0.121	1.90	108	59	9¼ “ “ “
	0.151	0.186	0.108	1.72	100	45	12 “ “ “
	0.158	0.163	0.151	1.08	97	43	14 “ “ “
	0.100	0.146	0.041	3.56	100	44	25 “ “ “

TABLE VII.

Dog B2-02.

Weight 10.5 kg. Very mild diabetes.

3 gm. glucose per kg. in 30 per cent solution by stomach tube.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.154	0.244	0.019	12.84	80	40	Blood before feeding.
4.50	0.196	0.270	0.080	3.37	82	39	1 hr. after “
1.85	0.149	0.159	0.133	1.12	77	38	2¾ hrs. “ “
Very faint.	0.093	0.112	0.062	1.80	75	38	4 “ “ “
0	0.094	0.112	0.067	1.67	80	40	7 “ “ “

The same conclusions hold for the mildly diabetic (Tables VII and VIII) and the severely diabetic (Tables IX and X) dogs. The last mentioned animal was found[6] in the preceding paper to have a very high renal threshold, but no comparable change in the permeability of the corpuscles for sugar was demonstrable.

TABLE VIII.

Mildly Diabetic Dogs on Starch Feeding.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
			Dog B2-43. Weight 10 kg. July 19, 1915.				
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0		0.133	0.103	1.29	70	43.5	Blood before feeding 100 gm. bread.
Faint.	0.137	0.178	0.087	2.04	96	45.0	2 hrs. after feeding.
0.53	0.189	0.222	0.133	1.66		37.1	5 " " "
			July 23.				
0	0.116	0.125	0.100	1.25	96	37.3	Blood before feeding 100 gm. bread.
0.98	0.172	0.217	0.095	2.28	93	37.0	3 hrs. after feeding.
			Dog B2-71. Weight 14 kg.				
0		0.125	0.112	1.11	90	27.2	Before feeding 200 gm. rice.
0.29	0.213	0.286	0.100	2.86	72		2 hrs. after feeding.
1.47	0.244	0.286	0.157	1.82		32.5	5 " " "

Table X gives the results of direct analyses of the corpuscles in comparision with the plasma after fat feeding in a severely diabetic animal. Though marked lipemia occurred, there was no appreciable alteration in the distribution of sugar between plasma and corpuscles.

TABLE IX.

Dog B2-79.

Weight 14.7 kg. Severe diabetes. 5 gm. glucose per kg. in 80 per cent solution by stomach tube.

Urine.								
Volume.	Glucose.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.105	0.105	0.105	1.00	108	47	Blood before injection.
6	Slight.	0.152	0.182	0.117	1.55	100	46	½ hr. after "
6	1.33	0.204	0.261	0.128	2.03	96	43	1 " " "
11	5.00	0.222	0.294	0.134	2.19	98	45	2 hrs. " "
11	5.90	0.182	0.294	0.078	3.77	114	52	3 " " "
No urine.		0.222	0.294	0.150	1.96	114	50	4 " " "
17	5.90	0.209	0.234	0.186	1.25	114	52	5½ " " "
14	3.50	0.208	0.238	0.180	1.32	114	52	7 " " "
10	2.64	0.228	0.332	0.143	2.32	116	55	9 " " "
10	1.67	0.222	0.333	0.106	3.14	115	49	10 " " "
12	0.53	0.200	0.260	0.149	1.74	108	54	12½ " " "
19	Faint.	0.165	0.194	0.135	1.43	113	49	16 " " "
25	"	0.145	0.168	0.117	1.43	112	45	18½ " " "
170	0	0.129	0.138	0.119	1.16	111	47	23½ " " "
95	0	0.120	0.133	0.102	1.30	90	42	25 " " "
70	0	0.118	0.143	0.083	1.72	90	42	26½ " " "
250	0	0.119	0.122	0.100	1.07	80	39	31½ " " "
63	0	0.133	0.143	0.119	1.20	107	39	35½ " " "
50	0	0.113	0.125	0.091	1.37	97	35	38½ " " "
125	0	0.100	0.112	0.078	1.43	94	36	45¾ " " "
40	0	0.080	0.098	0.053	1.84	96	40	50½ " " "

The totally depancreatized dog in Table XI had a noticeably high permeability of the corpuscles for sugar, both after the operation alone and after administration of 5 gm. of glucose per kilo by stomach. The ratios, nevertheless, varied with the usual unexplained irregularity, and there is no proof of any specific alteration due to pancreatectomy.

TABLE X.

Dog B2-79.

Severe diabetes. Fat feeding.

Date.	Urine sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Remarks.
1916	*per cent*	*per cent*	*per cent*		
Oct. 7	Faint.	0.182	0.075	2.42	Blood before feeding 300 gm. lard.
	0	0.186	0.109	1.70	4¼ hrs. after “
“ 9	0	0.118	0.076	1.55	Blood before “ 240 gm. suet.
	0	0.132	0.081	1.63	4 hrs. after “
“ 11	0	0.136	0.081	1.68	Blood before “ 360 gm. suet.
	0	0.166	0.112	1.48	4¾ hrs. after “
“ 14	0	0.137	0.125	1.09	Blood before “ 500 gm. suet.
	0	0.128	0.083	1.54	4 hrs. after “
“ 16	0	0.137	0.085	1.61	Blood before “ 100 gm. suet.
	0	0.154	0.085	1.81	5½ hrs. after “
“ 25	0	0.143	0.083	1.72	Blood before “ 230 gm. suet.
	0	0.143	0.083	1.72	3 hrs. after “

Tables XII to XVIII show the effect of exercise in a series of dogs ranging from non-diabetic to severely diabetic. The negative effect upon the permeability of the corpuscles to sugar is in harmony with the results of experiments upon human diabetics.[7]

---

[7] Allen, Stillman, and Fitz,[4] Chapter V.

TABLE XI.

Dog C3-01.

Weight 15 kg. Total pancreatectomy.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Volume.	Glucose.							
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.100	0.100	0.100	1.00	75	34	Blood before pancreatectomy.
		0.141	0.236	0.015 (?)	15.73	92	43	During ether anesthesia.
30	0.16	0.200	0.270	0.103	2.62	105	42	3¾ hrs. after operation.
65	1.05	0.250	0.270	0.220	1.22	107	40	5 " " "
70	2.18	0.238	0.282	0.184	1.53	101	45	7½ " " "
120	4.30	0.263	0.365	0.138	2.64	95	45	13½ " " "
57	2.59	0.200	0.227	0.159	1.42	87	40	16 " " "
60	2.00	0.244	0.314	0.129	2.43	99	38	18 " " "
110	4.79	0.270	0.270	0.270	1.00	93	38	21 " " "
113	3.94	0.227	0.256	0.175	1.46	86	36	23 " " "
150	5.18	0.312	0.408	0.125	3.26	82	34	26¾ " " "
100	5.00	0.334	0.500	0.063	7.93	81	38	29¾ " " "
110	5.86	0.334	0.400	0.206	1.94	80	34	32½ " " "
115	5.00	0.355	0.400	0.268	1.49	75	34	38½ " " "

5 gm. glucose per kg. in 30 per cent solution by stomach tube, 48 hrs. after operation.

90	4.27	0.244	0.358	0.050	7.10	83	37	Blood before feeding.
20	1.05	0.385	0.400	0.364	1.09	95	42	½ hr. after "
15	0.71	0.384	0.500	0.160	3.12	95	34	1 " " "
50	2.78	0.728	0.800	0.636	1.25	95	44	4 hrs. " "
65	0.16	0.666	0.910	0.367	2.47	80	45	8 " " "
No urine.		1.180	1.330	0.889	1.48	88	34	9 " " "
" "		0.800	1.140	0.140	8.14	88	34	12 " " "

The dog in Table XVIII had not only severe diabetes and a high renal threshold, but also marked acidosis. It is thus noticeable that acidosis had no demonstrable influence upon the sugar concentration in the corpuscles.

TABLE XII.

Dog B2-00.

Depancreatized just short of diabetes. Exercise. July 28, 1915. Fasting.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0.097	0.105	0.079	1.32	80	31.0	Blood before exercise.
0.097	0.115	0.057	2.01	89	31.2	After 1 hr. of exercise.
0.094	0.102	0.079	1.29	70	35.0	" 4 hrs. " "

Oct. 5. Exercise with 3 gm. glucose per kg. by stomach tube.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	**Hemoglobin.**	**Corpuscle volume.**	**Remarks.**
per cent	*per cent*	*per cent*		*per cent*	***per cent***	
0	0.128	0.084	1.52	110	43.0	**Blood before feeding and exercise.**
	0.182	0.081	2.24	100	45.0	**After ½ hr. of exercise and 40 min. after feeding.**
0	0.134	0.100	1.34	94	38.0	**After 1 hr. of exercise and 70 min. after feeding.**
0	0.200	0.109	1.83	88	40.5	**After 1 hr. of rest and 2 hrs. after feeding.**

In Tables XIX to XXI, cold had no perceptible influence upon the sugar content of the corpuscles in non-diabetic, mildly diabetic, and severely diabetic animals.

Table XXII shows the effect of intravenous glucose injections in a small adult horse. Table XXIII illustrates starch feeding

TABLE XIII.

Dog B2-01.

Depancreatized just short of diabetes. Exercise.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.

Fasting. July 28, 1915.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0.090	0.102	0.071	1.46	74	38.1	Blood before exercise.
0.114	0.143	0.058	3.46	78	34.2	After 1 hr. of exercise.
0.119	0.130	0.097	1.34	60	34.0	" 4 hrs. " "

Fasting. July 29.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
0.102	0.110	0.085	1.29	80	31.8	Blood before exercise.
0.095	0.100	0.086	1.16	81	36.0	After 2 hrs. of exercise.
0.103	0.135	0.046	2.93	73	35.9	" resting 4 hrs.

4 gm. glucose per kg. by stomach tube. Oct. 7.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.078	0.105	0.046	2.28	113	46.0	Blood before feeding and exercise.
	0.114	0.140	0.086	1.63	120	48.0	After 5 min. of exercise.
	0.130	0.168	0.091	1.84	120	49.5	" 15 " " "
	0.100	0.125	0.073	1.71	119	48.0	" ½ hr. " "
Slight.	0.125	0.147	0.084	1.75	111		" 1 " " "
Doubtful.		0.148	0.111	1.33			" 1 " " rest.

TABLE XIV.

Dog B2-02.

Very mild diabetes. July 13, 1915. 3 gm. glucose per kg. in 30 per cent solution subcutaneously.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0.53	0.102	0.143	0.058	2.46	102	48.3	Blood after 1 hr. of exercise and before injection.
5.00	0.202	0.242	0.154	1.57	106	45.5	After 2 hrs. of exercise.
1.11	0.109	0.119	0.095	1.25	80	41.2	" 3 " " "
1.00	0.095	0.097	0.092	1.05	100	45.0	" 5 " " "
0.50	0.133	0.167	0.076	2.20	87	37.5	" 8 " " " Fed bread and soup.
0	0.107	9.108	0.105	1.03	100	40.0	After resting all night.
0	0.096	0.100	0.090	1.11	100	41.0	" 1 hr. of heavy exercise.

TABLE XV.

Dog B2-43.

Mild diabetes. June 29, 1915. Fed 100 gm. bread.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.118	0.125	0.109	1.14	103	43.0	Blood before feeding and exercise.
0	0.106	0.118	0.090	1.31	83	43.2	After ½ hr. of hard exercise.
0	0.185	0.228	0.120	1.90	95	40.0	" 1¾ hrs. of rest.
0	0.091	0.091	0.091	1.00	98	40.5	" 2½ " " exercise.

July 26 and 27. Fed 50 gm. solid glucose.

Plasma sugar / Corpuscle sugar		Remarks.
Without exercise.	With exercise.	
1.3		Before feeding 50 gm. glucose.
1.6	2.5	1¼ hrs. after feeding.
1.4	1.4	3½ " " "
1.3	1.1	5 " " "

TABLE XVI.

Dog B2-63.

Mild diabetes. Bread feeding; Nov. 18, 1915, at rest; Nov. 19, with exercise.

Plasma sugar.		Corpuscle sugar.		Urine sugar.		Plasma sugar / Corpuscle sugar		Remarks.
Nov. 18.	Nov. 19.	Nov. 18.	Nov. 19.	Nov. 18.	Nov. 19.	Nov. 18.	Nov. 19.	
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
0.111	0.108	0.059	0.078	0	0	1.88	1.39	Before feeding. Exercise started.
0.127	0.095	0.087	0.087			1.46	1.09	½ hr. after feeding.
0.254	0.117	0.121	0.077	0	0	2.09	1.52	1 " " " Exercise stopped.
0.250	0.138	0.115	0.073	0.37	0	2.17	1.89	3 hrs. after feeding.
0.272	0.143	0.143	0.133	0.85	0	1.90	1.07	6 " " "

TABLE XVII.

Dog B2-79.

Severe diabetes. Comparison of days of exercise and rest, fasting.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.110	0.133	0.082	1.62	100	46.2	Before exercise July 17, 1915.
0	0.097	0.100	0.094	1.06	120	49.0	After 1 hr. of hard exercise.
0	0.102	0.109	0.093	1.17	105	45.3	Before exercise July 23.
0	0.093	0.105	0.080	1.31	125	49.5	After 3½ hrs. of exercise.
	0.115	0.169	0.061	2.77	110	50.0	Before exercise Sept. 25.
	0.128	0.143	0.114	1.25	110	52.0	After 1 hr. of exercise.

Feeding of 500 gm. beef lung; Nov. 8, at rest; Nov. 12, with exercise.

Plasma sugar / Corpuscle sugar		Remarks.
Nov. 8.	Nov. 12.	
1.2	1.6	Before feeding and exercise.
1.3	1.5	After 1 hr. of exercise.
1.4	1.1	" 2 hrs. " "
1.4	1.3	" 4 " " "

TABLE XVIII.

Dog B2-80.

Severe diabetes and acidosis. Exercise, fasting.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
Faint.	0.263	0.112	2.34	114	49.0	Blood before exercise July 23, 1915.
"	0.270	0.124	2.18	120	55.5	After 2½ hrs. of hard exercise.
0	0.208	0.124	1.68	118	50.5	Blood before exercise July 26.
0	0.208	0.129	1.61	125	53.6	After 1 hr. of hard exercise.
						Aug. 8.
0.65	0.294	0.155	1.39	102	55.0	Before exercise.
0.55	0.294	0.127	2.31	101	56.0	After 10 min. of exercise.
1.67	0.322	0.204	1.58	94	61.0	" resting 1⅓ hrs.
1.23	0.333	0.140	2.38	105	64.5	" ½ hr. of exercise.
1.09	0.303	0.187	1.62	104	53.4	" 4 hrs. " resting.

TABLE XIX.

Dog B2-00.

Depancreatized just short of diabetes.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
						Aug. 6, 1916. Cold, fasting.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.110	0.106	1.04	85	32.9	Blood before going in ice box.
0	0.085	0.063	1.35	82	35.7	After 2 hrs. in ice box.
0	0.098	0.092	1.06	78	33.5	" 19 " " " " Fed 12 hrs. ago.
	0.107	0.089	1.20	74	34.7	After 24 hrs. in ice box.
	0.125	0.046	2.72	70	30.7	" 2 " at room temperature.
	0.112	0.080	1.40	78		" 5½ " " " "
						Sept. 20, 1915. Cold and 3 gm. glucose per kg. by stomach tube.
0	0.154	0.071	2.07	107	42	Before feeding and going into ice box.
	0.271	0.109	2.48	104	46	1 hr. after feeding.
0	0.295	0.125	2.36	100	51	2 hrs. " "
0	0.100	0.071	1.41	110	44	4 " " "
0	0.098	0.084	1.17	100	47	7 " " "
0	0.110	0.076	1.45	100	38	19 " " "

and administration of glucose subcutaneously and by stomach in a goat. Tables XXIV and XXV show similar experiments in two sheep. The distribution of sugar between plasma and corpuscles in these species is seen to be not greatly different from that in the dog.

TABLE XX.

Dog B2-02.

Very mild diabetes. Cold and 3 gm. glucose per kg. by stomach tube.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.118	0.095	1.24	110	56.0	Before feeding and going in ice box.
Slight +.	0.228	0.115	1.98	104	54.0	1 hr. after feeding.
0	0.115	0.073	1.58	100	54.0	3 hrs. " "
0	0.100	0.065	1.54	100	52.2	6 " " "
	0.120	0.100	1.20	105	47.0	23 " " "

TABLE XXI.

Dog B2-79.

Severe diabetes. Feeding of 1 kg. beef lung, Nov. 23, 1915, in warm room, Nov. 30 in refrigerator.

Plasma sugar.		Corpuscle sugar.		$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$		Remarks.
Nov. 23.	Nov. 30.	Nov. 23.	Nov. 30.	Nov. 23.	Nov. 30.	
per cent	*per cent*	*per cent*	*per cent*			
0.133	0.250		0.200		1.25	Before feeding.
0.159	0.525	0.108	0.256	1.47	2.05	4 hrs. after feeding.
0.189	0.435	0.159	0.250	1.19	1.74	6 " " "

In the various preceding experiments, glucose and other foods were fed, and glucose also injected subcutaneously and intravenously, and no special differences of corpuscle permeability were found with the different modes of administration. Table XXVI shows the distribution of sugar between the plasma and corpuscles of sheep blood *in vitro* under 3 conditions: (*a*) Glucose was added to the oxalated blood *in vitro,* for observing its passage into the corpuscles; (*b*) the corpuscles were washed with 0.85 per cent NaCl solution and then suspended in this solution with addition of glucose, in order to test the influence of the saline upon the permeability; (*c*) the oxalated blood was diluted with the salt solution, so as to lower the concentration of sugar in the plasma and observe the passage of sugar out of the corpuscles. With reference to (*b*), the experiment of October 21 indicates that the salt solution altered the corpuscles in such a way as to cause them

TABLE XXII.

Normal Pony.

Weight 350 kg.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
Volume.	Glucose.						
Injection of 2 gm. glucose per kg. in 50 per cent solution intravenously. Nov. 11, 1917.							
cc.	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*	
	0	0.086	0.091	0.084	1.08	76	Blood before injection.
		0.825	1.250	0.305	4.10	45	5 min. after “
		0.446	0.675	0.258	2.61	55	15 “ “ “
432	5.40	0.391	0.568	0.287	1.98	63	½ hr. “ “
13	6.45	0.204	0.286	0.176	1.62	75	1½ hrs. “ “
			0.090	0.077	1.17		3½ “ “ “
		0.072	0.077	0.070	1.10	75	5 “ “ “
Same dose. Nov. 20.							
		0.115	0.125	0.112	1.09	78	Blood before injection.
		0.715	1.110	0.473	2.35	62	15 min. after “
1,240	5.05	0.500	0.625	0.446	1.70	70	½ hr. “ “
2,725	4.74	0.192	0.279	0.172	1.62	82	1½ hrs. “ “
		0.115	0.119	0.114	1.04	79	2½ “ “ “
640	2.15	0.064	0.056	0.066	0.08	80	4½ “ “ “

TABLE XXIII.

Goat 1.

Normal.

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
Apr. 10, 1917.						
	per cent	*per cent*	*per cent*		*per cent*	
10.20 a.m.	0.058	0.074	0.040	1.85		Fed pan of oats.
5.00 p.m.	0.058	0.084	0.014	6.00	37.5	6 hrs. after feeding.
Apr. 11.						
11.00 a.m.	0.047	0.068	0.012	5.66	37.7	12.30 p.m. Given subcutaneously 10 gm. glucose in 10 per cent solution.
1.30 p.m.	0.069	0.092	0.028	3.28	35.8	1 hr. after injection.
3.30 "	0.062	0.078	0.029	2.69	32.5	3 hrs. " "
6.00 "	0.056	0.069	0.033	2.09	36.0	
Apr. 18.						
2.45 p.m.	0.062	0.098	0.024	4.08	49.1	Given by stomach tube 100 gm. glucose in 30 per cent solution.
3.45 "	0.079	0.098	0.046	2.13	36.9	
4.45 "	0.056	0.085	0.009	9.44	34.5	
6.00 "	0.063	0.088	0.016	5.50	35.0	
8.00 "	0.049	0.056	0.039	1.44	41.2	
Apr. 23.						
11.30 a.m.	0.053	0.066	0.034	1.94		Given by stomach tube 300 gm. glucose in 10 per cent solution.
12.30 p.m.	0.097	0.137	0.069	1.98		
1.45 "	0.145	0.161	0.126	1.28	46.8	
3.30 "	0.119	0.152	0.071	2.13	41.0	Urine 98 cc. Sugar = 2.71 per cent.
4.30 "	0.130	0.133	0.126	1.05	42.3	
6.00 "	0.089	0.149	0.041	3.63	55.5	Urine 22 cc. Sugar = 6.05 per cent.

TABLE XXIV.

Sheep 1.

Normal.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar Corpuscle sugar	Corpuscle volume.	Remarks.
					Sept. 18, 1917.
per cent	*per cent*	*per cent*		*per cent*	
0.067	0.072	0.063	1.14	60	Blood before feeding oats and hay.
0.058	0.064	0.054	1.18	59	6 hrs. after feeding
					Sept. 20.
0.071	0.096	0.050	1.92	55	Blood before injecting 350 cc. 5 per cent glucose solution subcutaneously.
0.133	0.179	0.083	2.16	48	3 hrs. after injection.
0.125	0.154	0.093	1.66	48	5¾ " " "
					Sept. 21.
0.067	0.069	0.065	1.06	57	Blood before feeding 20 gm. glucose.
0.054	0.066	0.041	1.61	49	5 hrs. after feeding.
0.054	0.055	0.053	1.04	54	6 " " "
					Sept. 24.
0.065	0.071	0.060	1.18	55	Blood before feeding 50 gm. glucose.
0.065	0.069	0.060	1.15	46	4 hrs. after feeding.
0.077	0.080	0.072	1.11	41	5½ " " "
					Oct. 21
0.067	0.078	0.054	1.44	46	Blood before injecting 20 gm. glucose subcutaneously.
0.161	0.250	0.099	2.52	59	3½ hrs. after injection.
0.053	0.072	0.016	4.50	34	6½ " " "
					Oct. 26
0.071	0.079	0.046	1.72		Blood before injecting 20 gm. glucose subcutaneously.
0.079	0.102	0.055	1.85		3 hrs. after injection.
0.059	0.076	0.049	1.55		5½ " " "
					Oct. 29.
0.076	0.076	0.076	1.00		Blood before injecting 50 gm. glucose subcutaneously.
0.131	0.204	0.081	2.52		3½ hrs. after injection.
0.072	0.088	0.055	1.60		6 " " "

to contain less sugar. The result on November 14 was similar but less marked. The results under (*a*) and (*c*) showed the usual variability but on the whole resembled those *in vivo*, the corpuscle sugar concentration always running more or less below that of the plasma.

TABLE XXV.

Sheep 2.

Normal.

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Sept. 25, 1917.			
	per cent	*per cent*	*per cent*		*per cent*	
11.00 a.m.	0.054	0.062	0.043	1.44	43.3	Fed 50 gm. glucose.
12.00 m.	0.057	0.069	0.039	1.74	40.3	1 hr. after feeding.
2.00 p.m.	0.058	0.074	0.037	2.00	43.4	3 hrs. " "
4.00 "	0.058	0.056	0.060	0.93	49.3	5 " " "
			Oct. 22.			
11.30 a.m.	0.051	0.100	0.037	2.70		Fed 20 gm. glucose.
4.30 p.m.	0.070	0.085	0.054	1.57	48.0	5 hrs. after feeding.
			Oct. 23.			
11.00 a.m.	0.079	0.112	0.031	3.61	41.1	Fed 100 gm. glucose.
3.00 p.m.	0.076	0.112	0.017	6.58	37.8	4 hrs. after feeding.
5.00 "	0.062	0.074	0.050	1.48	38.3	6 " " "
			Dec. 28.			
9.00 a.m.	0.085	0.093	0.081	1.15	67.4	9.15 a.m. Given subcutaneously 43 gm. glucose in 12.5 per cent solution.
10.15 "	0.147	0.332	0.050	6.66	65.7	1 hr. after injection.
11.15 "	0.303	0.435	0.215	2.02	60.0	2 hrs. " "
12.15 p.m.	0.286	0.416	0.222	1.87	67.2	3 " " "
2.15 "	0.218	0.370	0.094	4.15	55.2	5 " " "

No attempt was made to determine the time required for passage of sugar into or out of the corpuscles *in vivo* or *in vitro*, but essentially the same ratios of distribution were found when the

TABLE XXVI.

Permeability of Sheep Corpuscles in Vitro.

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar.		Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Analyzed.	Calculated.			
			Sept. 18, 1918.				
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	
10.45 a.m.	0.067	0.072	0.065	0.064	1.1	60	To the remaining blood a pinch of glucose was added and allowed to stand at room temperature 1 hr.
11.45 "	0.880	0.880	0.880	0.880	1.0	55	
			Oct. 21.				
10.30 a.m.	0.067	0.078	0.041	0.054	1.9	46	To the remaining blood 0.1 gm. glucose was added and blood allowed to stand at room temperature 2 hrs.
12.30 p.m.	1.140	1.510	0.667	0.325	2.3	44	
12.30 "	0.910	1.510	0.286	0.098	5.2	43	Corpuscles of the 10.30 a.m. blood centrifuged, washed with salt solution, then made to 10 cc. with salt solution; 0.1 gm. glucose added and allowed to stand at room temperature 2 hrs.
2.00 "	0.161	0.250	0.084	0.098	3.0	59	Blood taken 4½ hrs. after subcutaneous injection of 50 gm. glucose. Remaining blood diluted ⅓ with salt solution and allowed to stand 1 hr.
3.00 "	0.082	0.132	0.062	0.056	2.1	33	

TABLE XXVI—*Concluded.*

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar.		Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Analyzed.	Calculated.			
Nov. 14.							
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	
11.30 a.m.	0.065	0.132	0.065	0.056	2.0	53	To the remaining blood 0.1 gm. glucose added and allowed to stand 2 hrs. at room temperature.
1.30 p.m.	2.00	2.78	1.35	1.41	2.1	57	
1.30 "	2.00	2.78	0.835	1.05	3.3	34	Corpuscles of 11.30 a.m. centrifuged and washed with salt solution, made to 10 cc. with salt solution, and 0.1 gm. glucose added.
3.00 "	0.169	0.217	0.062	0.122	3.5	51	3 hrs. after subcutaneous injection of 50 gm. glucose.
3.30 "	0.077	0.149	0.055		2.7	31	Remaining blood diluted ⅓ with salt solution and allowed to stand ½ hr.
4.00 "	0.114	0.118	0.056	0.106	2.1	34	Blood diluted ⅓ with salt solution after standing 1 hr.

intervals between analyses were 5 to 30 minutes, as in Tables IX, XI, and XXII, or several hours as in other tables. The observations suggest that the distribution of sugar between plasma and corpuscles is governed not by permeability in any proper sense but by a coefficient of solubility between the two media.

CONCLUSIONS.

1. No specific alteration of the distribution of sugar between the blood plasma and corpuscles was found in experiments with different quantities and modes of administration of glucose, different degrees of pancreatectomy and diabetes, lipemia, acidosis, exercise, cold, or different levels of the renal threshold.

2. The concentration of sugar in the corpuscles is normally a little below that in the plasma. The discrepancy in favor of the plasma generally becomes greater as the blood sugar rises. These relations hold good in the several animal species examined, and in experiments *in vivo* and *in vitro*. The statement that in the declining stage of hyperglycemia the corpuscles retain sugar longer than the plasma was not confirmed. The concentration in the corpuscles rarely equals that in the plasma, and the finding of an excess in the corpuscles is probably to be interpreted as an analytical error.

3. The term permeability as commonly used in relation to the sugar content of the corpuscles carries the idea that this is governed by a partially permeable membrane surrounding the corpuscle, and further that the solubility of glucose in the corpuscle substance is the same as in the plasma. This conception of permeability has received support from the assertions that the corpuscles take up and give off sugar more slowly than the plasma. It may possibly derive some support from the fact that washing in physiological salt solution changes the corpuscles in such a way that they take up less sugar, as reported by former authors. In view of the almost uniformly lower concentration in the corpuscles, however, and the quickness of adjustment on this basis whether the blood sugar rises or falls, it seems a more probable assumption that the coefficient of distribution depends upon solubility, glucose being more freely soluble in the plasma than in the corpuscle substance, perhaps because of the lipoid content of the latter.

4. Inasmuch as the sugar content of the corpuscles is subject to considerable irregularities from unknown causes and without known physiological significance, plasma analyses should be preferred to those of whole blood for experimental and clinical purposes.

THE ANTISCORBUTIC CONTENT OF CERTAIN BODY TISSUES OF THE RAT.

THE PERSISTENCE OF THE ANTISCORBUTIC SUBSTANCE IN THE LIVER OF THE RAT AFTER LONG INTERVALS ON A SCORBUTIC DIET.

By HELEN T. PARSONS.

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

(Received for publication, September 23, 1920.)

One of the most striking features of the need of the animal organism for the group of unidentified food factors is the great dissimilarity which certain animals exhibit in their requirements as contrasted with the great similarity existing among others even more widely separated in the biological classification. For example the guinea pig and the monkey have closely similar requirements for the antiscorbutic substance. On the other hand the two rodents, the guinea pig and the rat, differ most remarkably in this regard. A discrepancy of this kind cannot but challenge investigation, because any light which may be shed upon the matter may eventually offer a clue as to the nature of the function of the unidentified dietary essentials in animal nutrition, and since one of the main objects of animal experimental feeding is to obtain a basis for judging the nutritive requirements of other species with which experiments are not so easily conducted, an understanding of any such pronounced differences in requirements as this is certainly to be desired. Several possibilities suggest themselves to explain the phenomenon. The antiscorbutic substance may be normally absent from the tissues of the rat, because the rat may not need this substance for the normal functioning of its body. The rat may require antiscorbutic substance, but be capable of synthesizing it. The antiscorbutic substance may pass into a modified form in foods on ageing, drying, etc. and this form may not be utilizable by the guinea pig but be utilized by the rat. The rat's requirement for antiscorbutic substance may be strikingly low. The rat's requirement for antiscorbutic substance may approximate that of the guinea pig from the standpoint of metabolism, but there may be less waste of the substance due to a different rate of excretion, etc.

The problem has been approached by Harden and Zilva (1) and by Drummond (2) from the standpoint of the susceptibility of the rat to an antiscorbutic deficiency as exhibited by differences in growth when an antiscorbutic food was added to, or withheld from, a purified ration. Distinct differences were noted in favor of the rats given the antiscorbutic food and Drummond concludes:

> "It may therefore be accepted as experimentally proven that the dietary requirements of the higher animals include in addition to a satisfactorily balanced ration of proteins, fats, carbohydrate and mineral salts, an adequate supply of three accessory factors: (1) fat-soluble A, (2) water-soluble B, or antineuritic factor, (3) water-soluble C or antiscorbutic factor."

The following ration is one fed to rats by McCollum and Davis (3).

Ration 223 B.

	per cent
Wheat	64.0
Casein	13.4
Dextrin	12.8
Salt mixture	4.8
Butter fat	5.0

It will be observed that this ration is closely similar in its constituents to the purified rations of Harden and Zilva and Drummond except that the antineuritic factor is furnished by 64 per cent of wheat instead of by the yeast. Small amounts of the other dietary essentials furnished by the remaining purified constituents of this ration, are also furnished by the wheat. Fully as excellent growth was obtained in rats by feeding this ration as by the use of the purified rations to which antiscorbutic food was added. Reproduction was also excellent. It is necessary, therefore, to assume either that 64 per cent of wheat in this ration supplied the rat's antiscorbutic requirement as completely as the orange juice or the treated lemon juice in the other diets; or that some other essential in the diet was supplied by or improved by the wheat addition. Until it can be proved that every individual factor in the purified rations used by Harden and Zilva and by Drummond are optimum in kind and amount except the antiscorbutic factor, there may still be some room for doubt whether the improvement resulting from the addition of fairly large amounts of complex mixtures such as orange juice and treated lemon juice is in reality due to its antiscorbutic content.

Plan of the Experiment.

The present investigation was planned to approach the problem from the standpoint of determining the antiscorbutic content of the rat's body tissues, (1) when a typical scurvy diet was fed to the rat, (2) when the diet was high in antiscorbutic substance. It was planned to test the antiscorbutic content by feeding the rat tissues or extracts of the tissues to guinea pigs on a scorbutic diet. Investigators (4, 5) had reported negative results in obtaining any beneficial effect when meat juice was fed to guinea pigs, so that presumably the rat muscle tissue might be indecisive for the test. The amount of this factor in body organs was apparently not so well established. A few preliminary experiments were therefore run with beef liver and pig liver, and the relatively high content of these tissues in the antiscorbutic factor gave promise that the rat liver would prove suitable for the experiment. The rat muscle tissue was included in the experiment, however, because in the negative experiments reported by others the freshness of the meat was apparently not insured, and it was thought that since ageing plays such an important part in the destruction of the antiscorbutic substance under certain conditions, this factor alone might account for the discrepancy of results between guinea pig experiments and the experience of explorers and soldiers (4, 6, 7). Also there was a possibility that if the presence of the antiscorbutic factor in muscle was demonstrated there might be a more pronounced difference between the two sets of rats in regard to the amount contained in the muscle than in the case of an organ usually containing a larger amount. Since it was easy to obtain fresh fish in the local market it was thought to be of interest to feed fish muscle at the same level of intake. In testing for the antiscorbutic factor by means of guinea pig feeding it is more nearly satisfactory to add the material to be tested to the scorbutic diet from the beginning of the experiment and to determine its effectiveness in preventing scurvy than to try to overcome it after the syndrome has developed. This, however, requires so much material, except in instances where the antiscorbutic substance is practically absent, that in the present investigation it was thought necessary to test the material by determining its effectiveness in curing the symptoms of scurvy in the guinea pig. An arbitrary standard of 15 days of feeding the material to be tested was adopted, as this had been found sufficient to insure the disappearance of signs of scurvy when sufficient amounts of pig liver were fed. In some instances treatment during this full time interval was not adhered to because of the limited amount of material.

Production of Experimental Rats.

Young rats about one-third grown were selected and fed for a period of from 213 to 247 days (depending on the time of using the individual rat for the experiment) on a soy bean ration similar to that employed by Cohen and Mendel (8) and later modified by Givens and Cohen (9).

Scorbutic Ration I.

	per cent
Cooked soy bean meal	76.0
NaCl	3.0
Ca lactate	3.0
Dried yeast	3.0
Butter fat	5.0
Dried skimmed milk	8.0
Filter paper	2.0

This ration was selected because it was desired to use one which was adequate for the nutritive requirements of the rat over long periods of time, and which at the same time was a satisfactory diet on which to produce scurvy in the guinea pig. Guinea pigs refuse to consume a satisfactory amount of many of the complex rations on which the rat thrives, so that the range of suitable scurvy-producing diets for use in guinea pig feeding is therefore narrowed. During 136 days of the feeding period 13 per cent of milk solids was incorporated in the ration. 8 per cent of dried skimmed milk was used, together with 5 per cent of butter fat to replace the fresh milk used by these investigators. Attempts to autoclave the dried milk resulted in such a degree of caramelization that this treatment was abandoned. During 38 days at the beginning, and from 39 to 73 days at the end of the experiment (the variation due to the time of killing the animals), the dried milk was left out of the ration altogether, and replaced by an equivalent amount of soy bean meal to eliminate the slight source of antiscorbutic substance in the dried milk.

Scorbutic Ration II (Ration I without Dried Milk).

	per cent
Cooked soy bean meal	84.0
NaCl	3.0
Ca lactate	3.0
Dried yeast	3.0
Butter fat	5 0
Filter paper	2.0

Production of Control Rats.

The control set of rats were full grown, vigorous rats which had been discarded from various experimental rations in the rat colony, and for a period of 18 days were fed a stock ration with 5 cc. of orange juice per rat per day together with a water extract of the peel of oranges, added to the drinking water.

Feeding of Guinea Pigs.

Except in the cases where it was known that the guinea pigs had been fed on an abundance of succulent food immediately previous to the experiment they were given orange juice for a preliminary period of 2 days. A longer period was not deemed necessary since it was intended to produce scurvy in the guinea pig as a preliminary to testing the rat tissues. Scorbutic Ration II, described above, was used as the basal experimental diet. It had been found with many experiments with these soy bean rations that when the soy bean meal is heated just enough to destroy the raw taste but not enough to brown the product markedly, the ration was eaten greedily by the guinea pig, but it was found that even though the autoclave pressure and length of time of heating given by Cohen and Mendel were strictly adhered to, the results could be made to vary from this standard of cooking acceptable to the guinea pig palate, merely by varying the amount of meal that was put into the autoclave at one time, or the surface exposed, as in flat containers. This may account for some unsatisfactory results that have been reported by investigators when using this ration.[1] With this proper degree of cooking always maintained the ration is eaten so greedily that the growth curves become a very helpful index of the degree of scurvy in the animal, for the stationary or declining weight of the animal is often the first warning of the onset of the syndrome and improvement in the symptoms of scurvy is nearly uniformly accompanied by an improvement in the curve of growth, the correlation of the two probably not depending upon any hypothetical growth-promoting properties of the antiscorbutic, but upon the fact that the guinea pig ceases to eat this ration when the symptoms of scurvy make eating painful and resumes eating promptly when the symptoms subside.

Preparation of Material.

The rats were killed by chloroforming, the day the tissues were used for feeding. The muscles used were those easily removed from legs and back. In order to make the most economical use of the material and feed it at a high average level, it was necessary for the amount of muscle used to vary somewhat from day to day, since the rats were not of uniform size. The muscle was reduced to a fine pulp with shears and extracted with distilled water for 3 hours by standing at room temperature. The residue from this was extracted again with distilled water for approximately 22 hours by standing at ice box temperature. The quantity of water used was two-thirds the weight of the portion of muscle except in the case of the two highest levels of feeding muscle, where it was found necessary to reduce this quantity on certain days with a variation in the capacity of the guinea pig to consume these large amounts. In the case of the butterfish, as much of the muscle as possible was removed from the bones, 35 gm. of this were weighed out, and an extract was made similar to that of the rat muscle. These water extracts were fed by means of a glass syringe.

---

[1] Personal communication.

The given portions of the rat livers were weighed and rubbed through a wire sieve. This paste diluted somewhat with water could be readily fed from a glass syringe having a large opening. At times when the symptoms of scurvy in the guinea pigs were most severe it was necessary to strain out particles of the connective tissue with cheese-cloth as the least attempt at chewing appeared to produce much pain and a disinclination to take the material offered.

The results of the experiments are recorded in Table I and Charts I to VI.

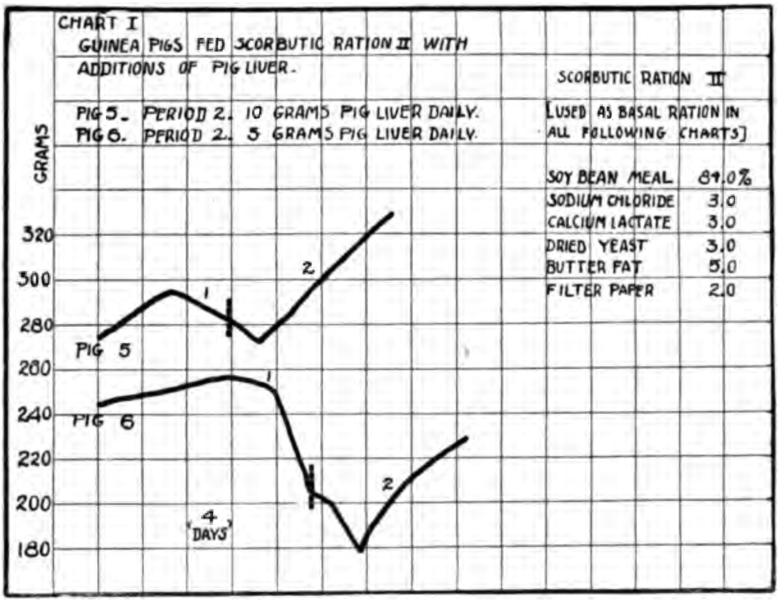


Chart I. The beneficial effects of feeding pig liver (Period 2) at sufficiently high levels to guinea pigs suffering from scurvy are shown. No. 5 on 10 gm. of liver per day made a complete recovery, but although improvement in the symptoms of scurvy of No. 6 was noted, apparently 5 gm. were not sufficient to insure the disappearance of the symptoms of scurvy in this time. The condition of scurvy in No. 6 was more severe than in No. 5, however, when the feeding of liver was begun. Although the pig liver was said to be fresh and not a cold storage product, the exact time that had elapsed since the killing of the animal could not be ascertained.

TABLE I.

Results of Experimental Diets.

No. of animal.	Day on which pronounced scurvy symptoms were noted.	Period described.	Tissues added to scorbutic diet of guinea pigs.				Day of experiment on which addition was begun.	No. of days addition was continued.	Remarks.	Autopsy.
			Animal from which taken.	Diet of this animal (or addition).	Tissue used.	Weight (or average weight) of tissues fed daily. *gm.*				
21	10	2	Rat.	Scorbutic Rations I and II.	Water extract of muscle.	34	10	6	No improvement in scurvy symptoms on adding muscle extract to diet. Died on 18th day.	Severe scurvy.
		3	Pig.		Liver.	12	16	2		
26	19*	2	Rat.	Scorbutic Rations I and II.	Water extract of muscle.	38	13	12	Adding extract did not prevent incipient scurvy from developing into severe scurvy. Chloroformed.	" "
24	20	2	"	Orange.	" " " "	37	18	7	No improvement in symptoms on adding small amount of extract. Great improvement with larger amount in Periods 3 and 4. Chloroformed.	Some evidences of scurvy still present. Imperfectly cured.
		3	"	"	" " " "	55		8		
		4	"	"	" " " "	95		7		
30	18	2	Butter-fish.		" " " "	35	18	10	No improvement in scurvy symptoms. Died on 28th day.	Severe scurvy.

5	10	2	Pig.		Liver.	10	12	14	Severe symptoms of scurvy disappeared on adding liver. Chloroformed.	Scurvy cured.
6	16	2	"		"	5	19	15	Very severe scurvy symptoms slightly improved on adding liver. Chloroformed.	Condition of scurvy still present.
20	10	2	Rat.	Scorbutic Rations I and II.	" kidney, spleen.	18	10	15	Severe symptoms of scurvy disappeared on adding organs. Chloroformed.	Scurvy cured.
31	15	2 3	" "	" " " "	Liver. "	10 5	13	4 10	Moderate symptoms of scurvy disappeared on adding liver. Chloroformed.	" "
33	17	2	"	" "	"	5	17	11	Severe symptoms of scurvy greatly improved on adding liver. Chloroformed.	Lesions of scurvy almost disappeared.
27	16	2	"	Orange.	"	5	16	15	Severe symptoms of scurvy disappeared on adding liver. Chloroformed.	Scurvy cured.
34	18	2	"	"	"	3	18	15	Severe symptoms of scurvy disappeared on adding liver. Chloroformed.	" "

* Scurvy symptoms not very pronounced.

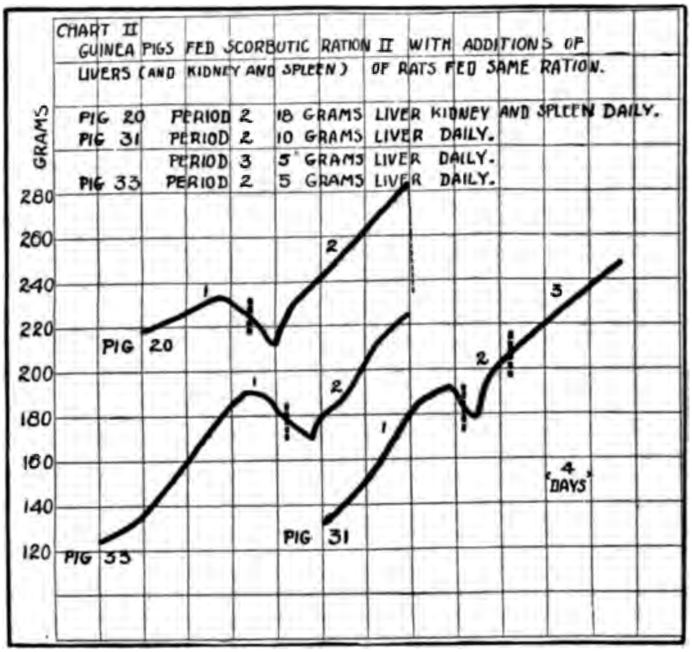


Chart II. This chart shows the beneficial effects (Periods 2 and 3) of feeding the livers of rats that had been fed a typical scurvy diet. In each of the three guinea pigs the symptoms of scurvy were severe and the recovery very rapid and marked. In the case of No. 20, the kidney and spleen of the rat were included with the liver to make the plane of intake higher, as it was very uncertain that there would be any considerable amount of the antiscorbutic substance in the tissues of the rats fed a diet in which it was so low. Towards the end of the experiment small rats were used and it was necessary to feed the organs of two rats instead of one on each alternate day to make the intake average 18 gm. daily. This high feeding on every other day produced on the last day of the experiment a digestive upset in the guinea pig with diarrhea, loss of appetite, and rapid loss in weight. No traces of scurvy appeared on autopsy, however, so that the symptoms had no relation to a condition of scurvy. Owing to the limited amount of material, No. 33 was fed liver for only 11 days. On autopsy slight traces of the scurvy condition were still present. These would undoubtedly have disappeared in 4 more days of feeding as completely as in No. 31 which received 10 gm. for 4 days and 5 gm. for the remaining 11 days. Apparently 5 gm. of this liver per day was not the minimum amount which would cure scurvy in the guinea pig.

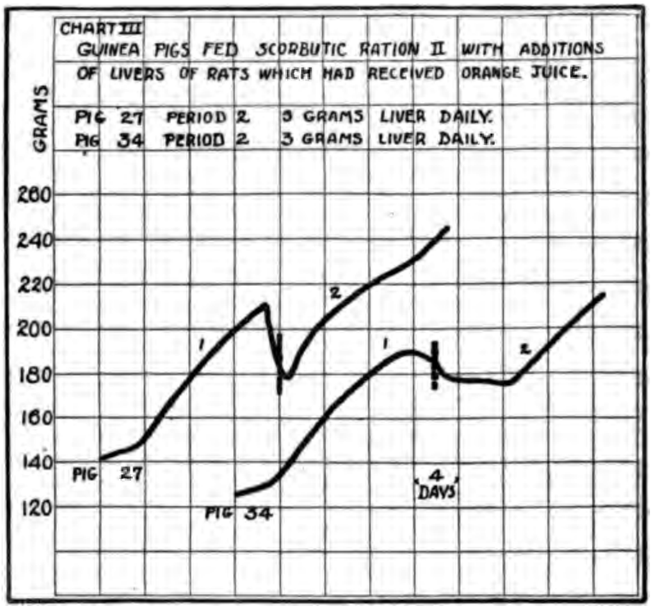


Chart III. The diet of these guinea pigs and those in Chart II are similar except that in Chart III the rats supplying the livers which were added during Period 2 were fed a large amount of antiscorbutic material in the form of orange juice for a period of 18 days previous to the experiment. A complete recovery was made in the case of both these guinea pigs but the recovery seemed to be somewhat slower in No. 34. Apparently 3 gm. of this liver a day is nearly the minimum amount which will produce a cure in this length of time. Compare these animals with those in Chart II where the rats supplying the livers had been fed the scorbutic soy bean rations.

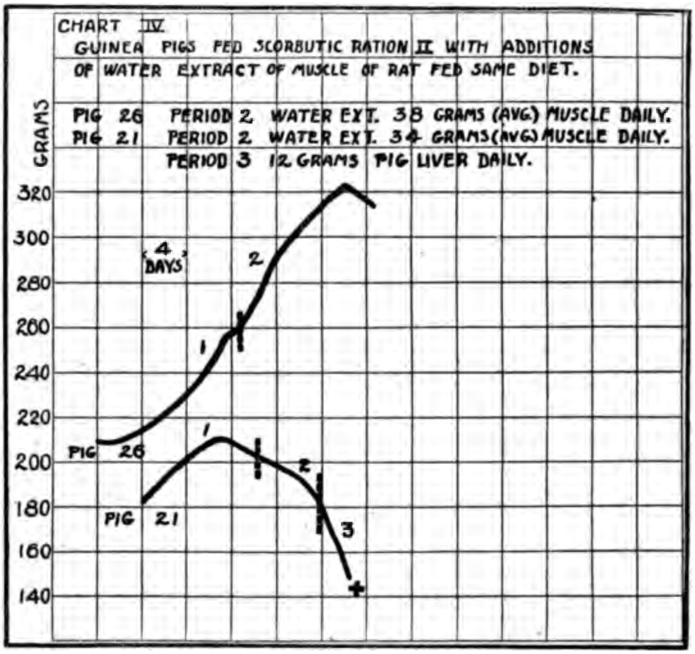


Chart IV. This chart illustrates the negative results in feeding guinea pigs water extract of the muscle of rats fed the soy bean diet. No. 21 was not benefited by 5 days of feeding this extract and was so weak and helpless at the end of this time that it took the extract with difficulty. An attempt was made to revive it by feeding pig liver but this failed and it died with severe scurvy.

No symptoms of scurvy had been observed when the feeding of No. 26 with this same water extract of muscle was begun, but these developed a few days later, and the guinea pig showed severe scurvy on autopsy. It was not possible to feed the muscle extract to this guinea pig at the same high level of intake as to No. 24 (Chart V), as it did not cooperate well in taking such large quantities.

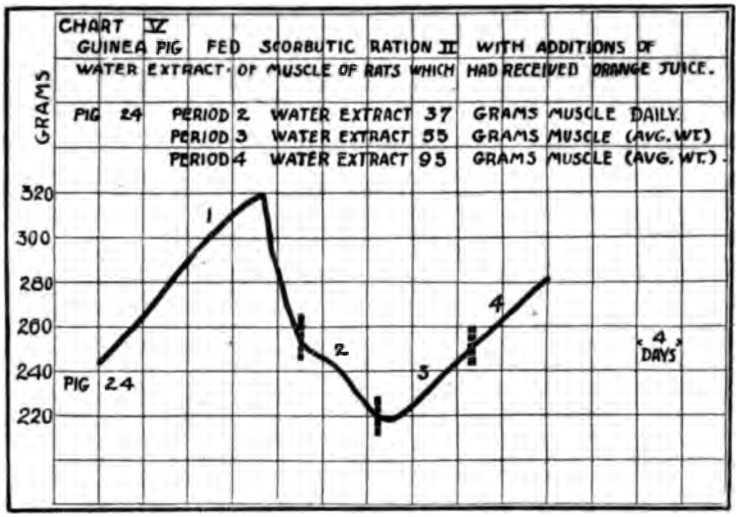


Chart V. This chart illustrates the effect of feeding a guinea pig water extract of muscle from a rat which had received orange juice. During the 7 days it was fed approximately the same level of intake that Nos. 21 and 26 received (Chart IV); the symptoms of scurvy grew steadily worse until the guinea pig was in a most miserable condition. However, it took the extract greedily so that it was possible to increase the amount fed. When the intake reached the equivalent of 55 to 95 gm. a day the symptoms improved, and the guinea pig was active and sleek when chloroformed. On autopsy, however, signs of scurvy were still present. Compare this chart with Chart IV.

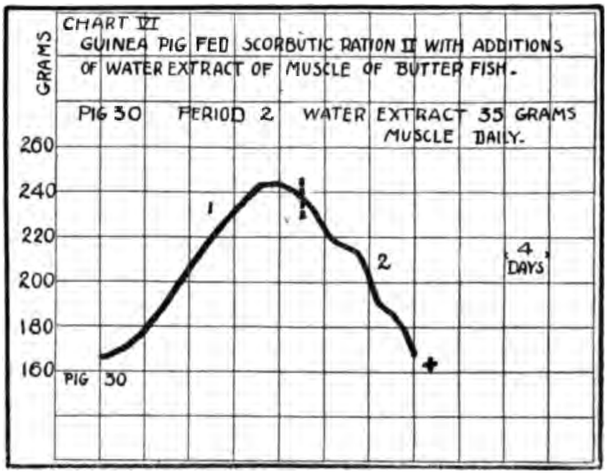


Chart VI. For purpose of comparison the water extract of fresh fish muscle was fed to this guinea pig at the same level at which extract of rat muscle was fed to No. 21. No improvement in the symptoms of scurvy was noted, but on the other hand the condition of scurvy grew steadily worse so that the pig died before the 15 days feeding was completed.

DISCUSSION.

The rapid disappearance of the symptoms of severe scurvy, substantiated by postmortem findings and correlated with the resumption of eating and a rapid gain in weight, during the 15 days (or less) when the guinea pigs were fed the livers of rats which had for a long period consumed a typical scurvy diet, leaves no doubt that this tissue with this history is rich in the antiscorbutic factor. It was, in fact, effective in relieving the symptoms of scurvy at such a low level of intake (5 gm. during a period of 11 days) that, without the use of larger numbers of animals, it could not be said that the livers of the control set of rats which had consumed orange juice were richer in this factor. This similarity between the two sets of rats in their tissue content of antiscorbutic factor is not contradicted by the results in feeding the muscle although the results in this case are indecisive, since it was not possible during this investigation to feed both at the same excessively high level. These results are in agreement with the instance cited by Smith (6) of the Arctic explorer, Dr. Kane, who attributed his freedom from scurvy during a winter on shipboard in the Arctic regions to the fact that he ate the rats with which the ship was infested. The diet upon which these rats lived must have been very low in the antiscorbutic substance.

This demonstration of such considerable amounts of the antiscorbutic factor in the livers of these rats disposes fairly definitely of the first hypothesis stated; namely, that the rat may not require antiscorbutic substance for its normal metabolism. It seems entirely improbable that the presence of this factor in the bodies of these animals should be accidental, especially since the amounts in the bodies of the two sets of rats approximate each other so closely. A synthesis of this factor is indicated, both by these results, and by the excellent growth and reproduction obtained in rats by the feeding of such rations as Ration 223 B cited above, in which no constituent has been shown to allay scurvy symptoms in the guinea pig. Closely allied to this hypothesis of a synthesis is the possibility that the rat can utilize some slightly different inactive forms of the antiscorbutic substance into which the active form might theoretically pass on ageing, drying, etc., and from which it might be rapidly changed in such physiological processes as the sprouting of seeds.

An alternative explanation is that the rat has a very definite food requirement for the antiscorbutic factor but that this is quantitatively very small in comparison with the requirement of the guinea pig and has been met in this experiment by the content of the soy bean rations used. This hypothesis might explain also the success of such rations as Ration 223 B. Some constituent of this diet might presumably carry a sufficient quantity of the antiscorbutic factor for the requirement of the rat, but escape detection in the light of the much larger requirement of the guinea pig. But if this explanation is accepted it is surprising to find such an abundance of the factor in the bodies of the rats fed on the scorbutic soy bean diet. It would be logical to assume that an extremely small requirement for a food factor would not necessitate the presence of a relatively large supply of that factor in the body of the animal. It is conceivable that the rat has acquired not only a lowered requirement but also a phenomenal capacity to store the antiscorbutic substance through a biological adaptation to a food supply which over long periods of time is very deficient in this substance. It is not apparent what relation the presence of this factor in the liver bears to its abundance or depletion in the rest of the body. It is conceivable that the liver cells are so constituted that a considerable supply of the antiscorbutic factor may be contained in them while at the same time the content of other tissues is below the level at which proper functioning is possible. It is proposed to test this point by determining also the antiscorbutic content of livers of guinea pigs suffering from scurvy as contrasted with the livers of normal guinea pigs. Until such a determination is made, the most probable hypothesis is to assume that such a strikingly large amount of the antiscorbutic factor as has been demonstrated in the livers of these rats indicates a satisfactory content in the body. It remains to be seen what amount of antiscorbutic substance is contained in the tissues of rats fed over long periods of time on adequate purified rations, freed from the antiscorbutic factor as thoroughly as can be done in the light of our present knowledge. This freedom is difficult to insure, as the guinea pig's requirement is so high that it is impossible to detect the presence of traces of antiscorbutic substance in their diet. In an otherwise purified diet the material carrying the water-soluble B might be under suspicion of also carrying traces of the antiscorbutic agent. The work of Delf (10) makes it uncertain also just what degree of heating would be necessary to insure that no trace of this substance would be left in a treated food.

Preliminary experiments of an investigation aimed to test the comparative amount of antiscorbutic substance in eggs and in newly hatched chickens appear to give some additional weight to the probability of a synthesis of antiscorbutic substance by certain animal tissues, or a utilization of forms other than those available to the guinea pig.

SUMMARY.

1. A high content of the antiscorbutic substance is demonstrated in the liver tissue of the rat not only after feeding for a short time on a diet rich in the antiscorbutic substance, but also after feeding over a long period of time on a typical scorbutic diet.
2. These results are offered as an indication of the need for the antiscorbutic factor in the normal metabolism of the rat.
3. Possible sources in the rat's body of the antiscorbutic substance, such as the presence of undemonstrated amounts in the food, utilization of a form not available to the guinea pig, and a synthesis of this factor by the rat's tissues, are discussed.

BIBLIOGRAPHY.

1. Harden, A., and Zilva, S. S., *Biochem. J.*, 1918, xii, 408.
2. Drummond, J. C., *Biochem. J.*, 1919, xiii, 77.
3. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615.
4. Chick, H., and Hume, E. M., *J. Roy. Army Med. Corps*, 1917, xxix, 121.
5. Dutcher, R. A., Pierson, E. M., and Biester, A., *Science*, 1919, l, 184.
6. Smith, A. H., *J. Roy. Army Med. Corps*, 1919, xxxii, 188.
7. Stefánsson, V., *J. Am. Med. Assn.*, 1918, lxxi, 1715.
8. Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.
9. Givens, M. H., and Cohen, B., *J. Biol. Chem.*, 1918, xxxvi, 127.
10. Delf, E. M., *Biochem. J.*, 1920, xiv, 211.

THE ANTISCORBUTIC REQUIREMENT OF THE PRAIRIE DOG.

By E. V. McCOLLUM and HELEN T. PARSONS.

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

PLATES 2 AND 3.

(Received for publication, September 27, 1920.)

In view of the fact that it is well demonstrated that certain species of mammals, as man, monkey, and guinea pig, cannot be adequately nourished on a diet which fails to supply a sufficient amount of the antiscorbutic substance (water-soluble C), whereas the rat can be nourished in a manner which at least closely approximates the normal during a long period without more than possible traces of antiscorbutic substance, it is of interest to examine other species in order to find how general among mammals is their susceptibility to scurvy.

We have, for example, seen young rats grow from weights of 40 to 60 gm. to essentially the full adult size on a diet of purified protein, dextrin, butter fat, a salt mixture, and agar-agar, supplemented with an alcoholic extract of 10 gm. of wheat germ per 100 gm. of ration to furnish the factor water-soluble B. One group of such rats was but slightly inferior in appearance after 15 months on this diet. The possibility is not excluded that these animals received a small amount of antiscorbutic substance from the wheat embryo extract, but their diet was one which would not have supplied sufficient antiscorbutic factor to prevent prompt development of scurvy in a guinea pig.

In the preceding paper Parsons[1] has discussed the problem of the source of the antiscorbutic factor in the livers of rats which were fed during long periods on diets which failed to afford any demonstrable protection against scurvy in a guinea pig. In the present communication we desire to describe an experiment which shows that another rodent, the western prairie dog (*Cynomys ludovicianus*), behaves like the rat rather than the guinea pig. The prairie dog is capable of growing during several months and remaining free from any symptoms of scurvy on a diet which utterly fails to protect the guinea pig against this disease.

---

[1] Parsons, H. T., *J. Biol. Chem.*, 1920, xliv, 587.

In January, 1918, seven prairie dogs were secured from New Mexico through the courtesy of the Bureau of Animal Industry, United States Department of Agriculture. They were, for want of better facilities, kept in laboratory cages in a heated building. Two litters of young were secured, one of which was lost through accidental injuries to the mother. Two young from another litter were successfully weaned. These were employed as subjects in the experiment described in this paper. One died from digestive disturbances soon after the beginning of the experiment.

Our purpose in using these animals for this study was to compare them with guinea pigs in respect to their susceptibility to scurvy. We sought young prairie dogs for this study because, like the rat and guinea pigs, they are rodents, and of approximately the same size as the guinea pig. Furthermore, the diet of the prairie dog, at least during a large part of the year, consists of succulent grass rich in antiscorbutic substance.

The animal which grew to maturity, and the growth curve of which is presented in Chart 1, weighed 18 gm. when 1 day old, and at the age of 62 days weighed 169 gm. At this age (Period 2) it was placed on a modification of the soy bean flour diet found by Cohen and Mendel[2] to be satisfactory for inducing growth in young guinea pigs when supplemented with a suitable source of the antiscorbutic substance, but incapable without such addition of protecting the animals against the prompt development of scurvy. The diet used was essentially like that of Cohen and Mendel, except that it was poorer in antiscorbutic substance. They included in their diet sufficient rich Jersey milk to furnish 5 per cent butter fat. We replaced the milk in our diet by 5 per cent purified butter fat and soy bean flour.

Although this diet would lead to the development of scurvy in the guinea pig in 2 to 3 weeks, the young prairie dog in our

---

[2] Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

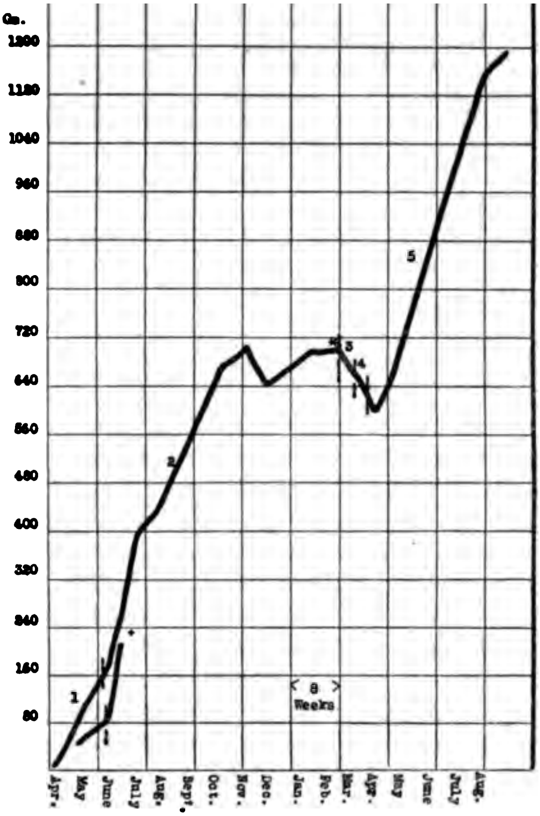


CHART 1. The long growth curve in the chart shows the history of a young prairie dog. Period 1 represents the period of nursing, to independence in diet. In Period 2 the animal was fed a diet consisting of cooked soy bean meal 84.0, sodium chloride 3.0, calcium lactate 3.0, dried yeast 3.0, butter fat 5.0, and filter paper 2.0 per cent. This diet contains too little antiscorbutic substance to be demonstrated with a guinea pig. On this diet the animal grew during 6 months from a weight of 169 to 690 gm. After this time there was very little growth on this diet. There is the possibility that the hibernating instinct was in part responsible for the cessation of growth which occurred about December 1.

The animal was photographed at the point marked by an asterisk. In order to determine whether the cessation of growth was due to lack of the antiscorbutic factor in the food, 7 to 9 gm. of peeled orange were given daily during 3 weeks, but the animal declined 40 gm. in weight during the period. In Period 4 the orange was omitted and 1 per cent cod liver oil was introduced into the diet. This modification did not arrest decline. In Period 5 the diet was changed to a mixture of grains and dried buttermilk, and after 4 days 5 gm. of green grass were given every few days. On this diet the prairie dog began promptly to gain in weight and continued to grow. During the following 6 months the animal grew from 604 to 1,195 gm.

The behavior of the animal in Period 2 on a scorbutic diet shows that this species is able to grow rapidly from nine to twelve times the interval necessary for the development of scurvy in the guinea pig on the same diet. At no time during the experiment did the prairie dog manifest any symptoms of scurvy.

experiment grew well on it during a period of 6 months. At the end of this time it showed no signs of scurvy but became lethargic and had a poor appetite. This was probably due in part at least to its hibernating instinct, for the cessation of growth happened about December 1. By March 1, however, the animal was awake and active, but did not resume growth. In order to discover whether this could be due to the lack of the antiscorbutic substance it was given 7 to 9 gm. daily of peeled orange. It did not at this time show any symptoms of scurvy. It declined about 40 gm. in weight during 3 weeks while receiving the orange, which seemed sufficient evidence that the decline was not due to the lack of this dietary factor. The orange was then discontinued, and 1 per cent of cod liver oil was included in the diet during the next 12 days, but this change did not arrest the decline. The animal was then changed to a ration consisting of mixed grains and dried buttermilk, and was given small amounts of green grass at intervals of a few days. It began to increase in weight within a week, and at the age of nearly 19 months weighs 1,195 gm., whereas it weighed 700 gm. at its highest point reached while fed the scorbutic diet. At the time of writing this paper (September 21) the animal is again beginning to appear drowsy and takes less than the usual amount of food.

The following explanations might be brought forward to account for the arrest in growth about December 1: (1) Lack of antiscorbutic substance; (2) instinct to hibernate at this season of the year; and (3) some other fault in the diet.

The first of these possible explanations seems satisfactorily disposed of by the failure of the animal to respond to 3 weeks feeding of orange juice.

Givens and Cohen[3] found a diet similar to that of Cohen and Mendel, consisting of soy bean flour 76.3; milk solids 11.4; dried yeast 2.9; paper 2.9; calcium lactate 2.9; sodium chloride 2.9 per cent, to be satisfactory for the growth of rats to maturity, but these rats failed to rear the young which were born. There can be no doubt that the food supply of our prairie dog, which was similar to their diet except that about 7 to 8 per cent of the nonfat solids of the milk was replaced by soy bean flour, was inferior in its nutritive value to theirs. This substitution was made for the purpose of eliminating a known source of antiscorbutic substance.

---

[3] Givens, M. H., and Cohen, B., *J. Biol. Chem.*, 1918, xxxvi, 127.

The record of our prairie dog is of interest because it shows that this species is capable of growing for a period of 6 months on a scorbutic diet. This is equivalent to nine to twelve times the interval that is necessary for the development of scurvy in the guinea pig.

EXPLANATION OF PLATES.

PLATE 2.

FIGS. 1 and 2. The photographs were taken February 29, 1920. The animal had then been on the scorbutic diet 263 days. About December 1 it ceased to grow, but remained in good condition and at its maximum weight up to the time of taking these pictures. These photographs, therefore, show the excellent nutrition of the animal about the time when orange juice was introduced in the diet.

PLATE 3.

FIGS. 3 and 4. These photographs were taken September 19, 1920, after the animal had been on a mixed diet containing small amounts of grass for 153 days. Fig. 4 shows a marked accumulation of fat. This is probably sufficient to account for most of the gain in weight which followed the change from the scorbutic diet to the mixed diet containing the grass.

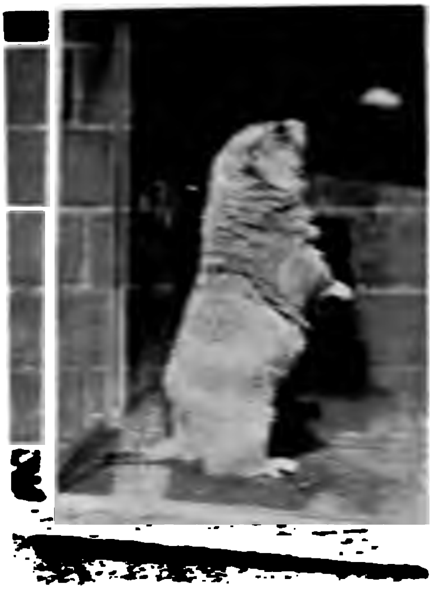


Fig. 1.

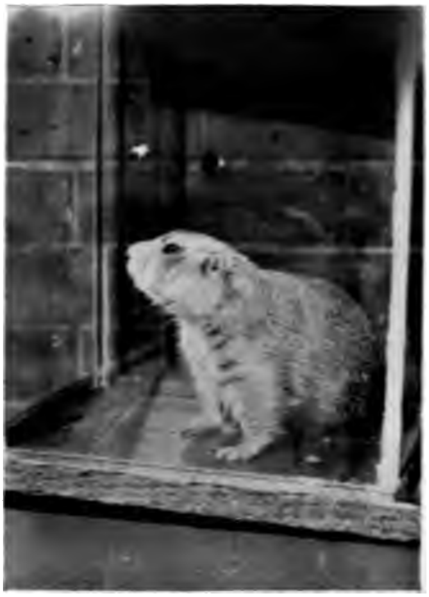


Fig. 2.

(McCollum and Parsons: Antiscorbutic requirement of prairie dog

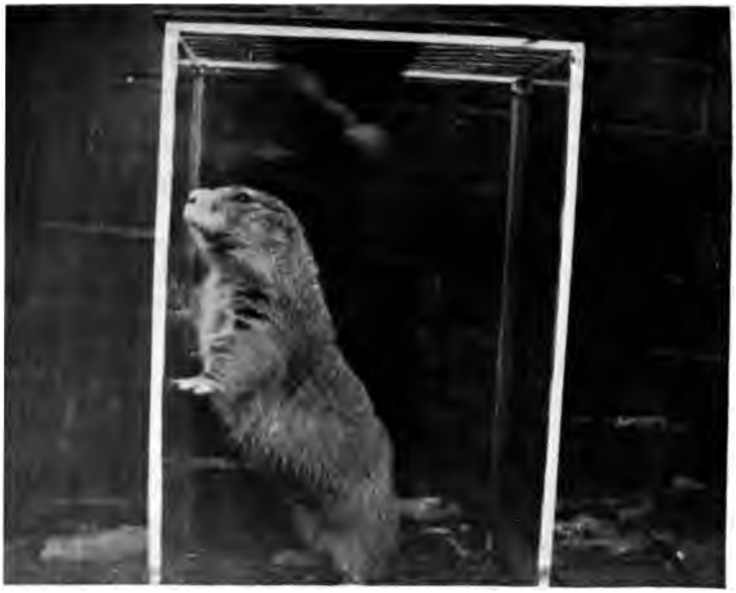


FIG. 3.

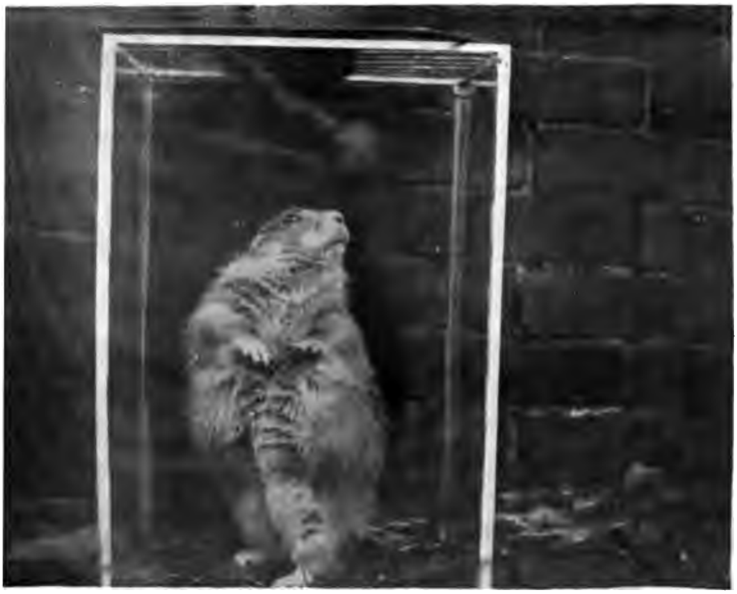


FIG. 4.

(McCollum and Parsons: Antiscorbutic requirement of prairie dog.)

INDEX TO VOLUME XLIV.

ACETALDEHYDE, production by certain pentose-fermenting bacteria, 29

Acetone production by *Bacillus acetoethylicum*, certain influencing factors, 465

Acid, carbonic, and sodium bicarbonate (CO_2 ratio), alteration in the blood during elevation of body temperature, 131

——, inosite phosphoric, of plants, 429

Acids, amino-, from the globulin of the coconut as determined by the butyl alcohol extraction method of Dakin, 283

——, ——, of gelatin, 499

Alcohol, butyl, extraction method of Dakin, determination of some amino-acids from the globulin of the coconut, 283

Aldrich, Martha. See Denis and Aldrich, 203

Alkali reserve of marine fish and invertebrates. The excretion of carbon dioxide, 329

Alkaline reserve of the body, attempt toward the determination, 211

Amino-acids from the globulin of the coconut as determined by the butyl alcohol extraction method of Dakin, 283

—— of gelatin, 499

Anderson, R. J. Composition of inosite phosphoric acid of plants. Seventeenth paper on phytin, 429

Anemia, acute, lipemia, 363

Antiberi-beri vitamine, a test and its practical application, 487

Antineuritic substance, study of the factors which interfere with the use of yeast as a test organism, 113

Antiscorbutic content of certain body tissues of the rat, 587

—— requirement of the prairie dog, 603

—— substance, persistence in the liver of the rat after long intervals on a scorbutic diet, 587

Arzberger, C. F., Peterson, W. H., and Fred, E. B. Certain factors that influence acetone production by *Bacillus acetoethylicum*, 465

Autolysis, effect of certain colloids, 553

—— studies, 553

BACILLUS acetoethylicum, certain factors that influence acetone production, 465

Bacteria, production of acetaldehyde by certain pentose-fermenting, 29

Bailey, C. H., and Gurjar, A. M. Respiration of cereal plants and grains. II. Respiration of sprouted wheat, 5 III. Respiration of rice paddy and milled rice, 9 IV. The respiration of frosted wheat plants, 13 V. Note on the respiration of wheat plants infected with wheat rust, 17

Bean, mung, *Phaseolus aureus* Roxburgh, some proteins from, 303

Bean, velvet, dihydroxyphenylalanine, a constituent of, 481

Beans, navy, and carrots, effect of cooking on the water-soluble vitamine, 159

Bell, Richard D., and **Doisy, Edward A.** Rapid colorimetric methods for the determination of phosphorus in urine and blood, 55

Blood, alteration of the CO_2 ratio (H_2CO_3:$NaHCO_3$) during elevation of body temperature, 131

—— and urine, rapid colorimetric methods for the determination of phosphorus, 55

—— corpuscles, permeability to sugar, 563

——, diastatic ferments, 455

——, estimation of chlorides, 47

—— fat, 345, 363

—— —— constituents of rabbits under normal conditions, variation, 345

——, note on the preservation of specimens intended for blood sugar determinations, 203

Bodansky, Meyer. Biochemical studies on marine organisms. The occurrence of zinc, 399

——. See **Rose** and **Bodansky**, 99

Body, attempt toward the determination of the total alkaline reserve, 211

—— temperature, alteration of the CO_2 ratio (H_2CO_3:$NaHCO_3$) in the blood during elevation, 131

—— tissues of the rat, antiscorbutic content, 587

Bradley, H. C., and **Felsher, H.** Studies of autolysis. VI. Effect of certain colloids upon autolysis, 553

Brain tissue, freed from cholesterol, influence upon the growth of the white mouse, 439

Butyl alcohol extraction method of Dakin, determination of some amino-acids from the globulin of the coconut, 283

CABBAGE and onion, water-soluble B, 175

Calcium requirement of maintenance in man, 21

Cameron, A. T., and **Hollenberg, M. S.** The nature of chlorine combination in urine, 239

Carbohydrate metabolism and diabetes, experiments, 563

Carbon dioxide excretion, effect on alkali reserve of marine fish and invertebrates, 329

—— —— ratio (H_2CO_3:$NaHCO_3$) in the blood during elevation of body temperature, alteration, 131

Carbonic acid and sodium bicarbonate (CO_2 ratio), alteration in the blood during elevation of body temperature, 131

Carrots and navy beans, effect of cooking on the water-soluble vitamine, 159

Cereal plants and grains, respiration, 5, 9, 13, 17

Chlorides in blood, estimation, 47

Chlorine combination in urine, 239

Cholesterol content of the serum in pneumonia, variation, 215

——, influence upon the growth of the white mouse of brain tissue freed from, 439

Coconut, amino-acids from the globulin as determined by the butyl alcohol extraction method of Dakin, 283

——, *Cocos nucifera*, hydrolysis of the globulin, 291

Cocos nucifera, coconut, hydrolysis of the globulin, 291

Collip, J. B. The alkali reserve of marine fish and inverte-

brates. The excretion of carbon dioxide, 329
Colloids, certain, effect upon autolysis, 553
Colorimetric methods, rapid, for the determination of phosphorus in urine and blood, 55
Conklin, Ruth E. See Mattill and Conklin, 137
Copper occurrence in marine organisms, 99
Corn, skimmed milk as a supplement in feeding, 1
Corpuscles, blood, permeability to sugar, 563

DAKIN, H. D. Amino-acids of gelatin, 499
Dakin's butyl alcohol extraction method, determination of some amino-acids from the globulin of the coconut, 283
Daniels, Amy L., and Loughlin, Rosemary. A deficiency in heat-treated milks, 381
Denis, W., and Aldrich, Martha. Note on the preservation of specimens of blood intended for blood sugar determinations, 203
Determination of chlorides in blood, 47
—— —— phenolic substances in urine, 409
—— —— phosphorus in urine and blood, rapid colorimetric methods, 55
—— —— some amino-acids from the globulin of the coconut by the butyl alcohol extraction method of Dakin, 283
—— —— total alkaline reserve of the body, attempt, 211
—— —— vitamine, quantitative method in connection with determinations of vitamine in glandular and other tissues, 531
Determinations, blood sugar, note on the preservation of blood intended for, 203
Diabetes and carbohydrate metabolism, 563
Dialysis or extraction, continuous, apparatus, 207
Diastase in the sweet potato in relation to the preparation of sweet potato syrup, 19
Diastatic ferments of the blood, 455
Diet, effect on the excretion of indican and the phenols, 69
——, scorbutic, persistence of the antiscorbutic substance in the liver of the rat after long intervals on, 587
Diets deficient in water-soluble (B) vitamine, metabolism studies, 277
Dihydroxyphenylalanine, a constituent of the velvet bean, 481
Doisy, Edward A. See Bell and Doisy, 55
Dubin, Harry E. See Funk and Dubin, 487

EMBRYONIC livers, fat content, 243
Excretion of carbon dioxide, effect on alkali reserve of marine fish and invertebrates, 329
—— —— indican and the phenols, effect of diet, 69
Extraction method of Dakin, butyl alcohol, determination of some amino-acids from the globulin of the coconut, 283
—— or dialysis, continuous, apparatus, 207

FAT, blood, 345, 363
——, ——, constituents of rabbits under normal conditions, variation, 345

Fat content of embryonic livers, 243

Feeding, skimmed milk as a supplement to corn, 1

Felsher, H. See Bradley and Felsher, 553

Ferments of the blood, diastatic, 455

Fred, E. B. See Arzberger, Peterson, and Fred, 465

——. See Peterson and Fred, 29

Funk, Casimir, and Dubin, Harry E. A test for antiberi-beri vitamine and its practical application, 487

GELATIN amino-acids, 499

Glandular and other tissues, quantitative method for determination of vitamine in connection with determinations of vitamine in, 531

Globulin of the coconut, amino-acids as determined by the butyl alcohol extraction method of Dakin, 283

—— —— —— ——, *Cocos nucifera*, hydrolysis, 291

Gore, H. C. Occurrence of diastase in the sweet potato in relation to the preparation of sweet potato syrup, 19

Graham, S. G. See Imrie and Graham, 243

Grains and cereal plants, respiration, 5, 9, 13, 17

Growth, experimental studies, 439

—— of the white mouse, influence of brain tissue, freed from cholesterol, 439

Gurjar, A. M. See Bailey and Gurjar, 5, 9, 13, 17

HAGGARD, Howard W. Hemato-respiratory functions. VI. The alteration of the CO_2 ratio (H_2CO_3:$NaHCO_3$) in the blood during elevation of body temperature, 131

Harbo, H. G. See Prentice, Lund, and Harbo, 211

Hart, E. B., and Humphrey, G. C. Can "home grown rations" supply proteins of adequate quality and quantity for high milk production. II, 189

Heat-treated milks, deficiency, 381

Hemato-respiratory functions, 131

Hollenberg, M. S. See Cameron and Hollenberg, 239

Horiuchi, Yajiro. Studies on blood fat. I. Variation of the blood fat constituents of rabbits under normal conditions, 345 II. Lipemia in acute anemia, 363

Humphrey, G. C. See Hart and Humphrey, 189

Hydrolysis of the globulin of the coconut, *Cocos nucifera*, 291

IMRIE, C. G., and Graham, S. G. The fat content of embryonic livers, 243

Indican and the phenols, effect of diet on the excretion, 69

Inosite phosphoric acid of plants, 429

JOHNS, Carl O., and Jones, D. Breese. Some amino-acids from the globulin of the coconut as determined by the butyl alcohol extraction method of Dakin, 283

——, and Waterman, Henry C. Some proteins from the mung bean, *Phaseolus aureus* Roxburgh, 303

——. See Jones and Johns, 291

Jones, D. Breese, and Johns, Carl O. Hydrolysis of the globulin of the coconut, *Cocos nucifera*, 291

——. See Johns and Jones, 283

KARR, Walter G. Metabolism studies with diets deficient in water-soluble (B) vitamine, 277
——. Some effects of water-soluble vitamine upon nutrition, 255
Kipp, Harold A. Variation in the cholesterol content of the serum in pneumonia, 215

LEWIS, D. Sclater, and Mason, Edward H. The diastatic ferments of the blood, 455
Lipemia in acute anemia, 363
Liver of the rat, persistence of the antiscorbutic substance after long intervals on a scorbutic diet, 587
Livers, fat content of embryonic, 243
Loughlin, Rosemary. See Daniels and Loughlin, 381
Lund, H. O. See Prentice, Lund, and Harbo, 211
Lynch, Vernon. Chemistry of the whitefish sperm, 319

MAINTENANCE in man, calcium requirement, 21
Mann, Hubert. An apparatus for continuous dialysis or extraction, 207
Mason, Edward H. See Lewis and Mason, 455
Mattill, H. A., and Conklin, Ruth E. The nutritive properties of milk, with special reference to reproduction in the albino rat, 137
McCollum, E. V., and Parsons, Helen T. The antiscorbutic requirement of the prairie dog, 603
——. See Souza and McCollum, 113
Mendel, Lafayette B. See Osborne and Mendel, 1
Metabolism, carbohydrate, and diabetes, experiments, 563
—— studies with diets deficient in water-soluble (B) vitamine, 277
Method of Dakin, butyl alcohol extraction, determination of some amino-acids from the globulin of the coconut, 283
——, quantitative, for the determination of vitamine in connection with determinations of vitamine in glandular and other tissues, 531
Methods, rapid colorimetric, for the determination of phosphorus in urine and blood, 55
Milk, nutritive properties, with special reference to reproduction in the albino rat, 137
—— production, high, can "home grown rations" supply proteins of adequate quality and quantity, 189
——, skimmed, as a supplement to corn in feeding, 1
Milks, deficiency in heat-treated, 381
Miller, Elizabeth W. The effect of cooking on the water-soluble vitamine in carrots and navy beans, 159
Miller, Emerson R. Dihydroxyphenylalanine, a constituent of the velvet bean, 481
Myers, Victor C., and Short, James J. The estimation of chlorides in blood, 47

NUTRITION, some effects of water-soluble vitamine, 255
Nutritive properties of milk, with special reference to reproduction in the albino rat, 137

ONION and cabbage, water-soluble B, 175

Osborne, Thomas B., and Mendel, Lafayette B. Skimmed milk as a supplement to corn in feeding, 1

Parsons, Helen T. The antiscorbutic content of certain body tissues of the rat. The persistence of the antiscorbutic substance in the liver of the rat after long intervals on a scorbutic diet, 587
——. See McCollum and Parsons, 603
Pentose-fermenting bacteria, production of acetaldehyde, 29
Permeability of blood corpuscles to sugar, 563
Peterson, W. H., and Fred, E. B. The production of acetaldehyde by certain pentose-fermenting bacteria, 29
——. See Arzberger, Peterson, and Fred, 465
Phaseolus aureus Roxburgh, mung bean, some proteins from, 303
Phenolic substances in urine, estimation, 409
Phenols and indican, effect of diet on the excretion, 69
Phosphoric, inosite, acid of plants, 429
Phosphorus in urine and blood, rapid colorimetric methods for the determination, 55
Phytin, 429
Pneumonia, variation in the cholesterol content of the serum, 215
Potato, sweet, occurrence of diastase in relation to the preparation of sweet potato syrup, 19
Prentice, W., Lund, H. O., and Harbo, H. G. An attempt toward the determination of the total alkaline reserve of the body, 211
Preservation of specimens of blood intended for blood sugar determinations, note, 203
Proteins from the mung bean, *Phaseolus aureus* Roxburgh, 303
—— of adequate quality and quantity for high milk production, can "home grown rations" supply, 189

Ray, L. A. See Robertson and Ray, 439
Reproduction in the albino rat, nutritive properties of milk, with special reference to, 137
Reserve, alkali, of marine fish and invertebrates. The excretion of carbon dioxide, 329
——, total alkaline, of the body, attempt toward determination, 211
Respiration of cereal plants and grains, 5, 9, 13, 17
—— —— frosted wheat plants, 13
—— —— rice paddy and milled rice, 9
—— —— sprouted wheat, 5
—— —— wheat plants infected with stem rust, note, 17
Respiratory-hemato functions, 131
Rice paddy and milled rice, respiration, 9
Robertson, T. Brailsford, and Ray, L. A. Experimental studies on growth XVI. The influence of brain tissue, freed from cholesterol, upon the growth of the white mouse, 439
Rose, William C., and Bodansky, Meyer. Biochemical studies on marine organisms. I. The occurrence of copper, 99
Rust, stem, note on the respiration of wheat plants infected, 17

SCORBUTIC diet, persistence of the antiscorbutic substance in the liver of the rat after long intervals on, 587

Serum in pneumonia, variation in the cholesterol content, 215

Sherman, H. C. Calcium requirement of maintenance in man, 21

Short, James J. See Myers and Short, 47

Simpson, George Eric. See Underhill and Simpson, 69

Sodium bicarbonate and carbonic acid (CO_2 ratio), alteration in the blood during elevation of body temperature, 131

Souza, Geralda de Paula, and McCollum, E. V. A study of the factors which interfere with the use of yeast as a test organism for the antineuritic substance, 113

Sperm, chemistry of the whitefish, 319

Sugar, blood, note on the preservation of specimens of blood intended for determinations, 203

——, permeability of blood corpuscles to, 563

Swoboda, Frederick K. A quantitative method for the determination of vitamine in connection with determinations of vitamine in glandular and other tissues, 531

TEMPERATURE, body, alteration of the CO_2 ratio (H_2CO_3: $NaHCO_3$) in the blood during elevation, 131

Test for antiberi-beri vitamine and its practical application, 487

—— organism, study of factors which interfered with the use of yeast for the antineuritic substance, 113

Tisdall, Frederick F. Estimation of the phenolic substances in urine, 409

Tissue, brain, freed from cholesterol, influence upon the growth of the white mouse, 439

Tissues, body, of the rat, antiscorbutic content, 587

——, glandular and other, quantitative method for determination of vitamine in connection with determinations of vitamine in, 531

UNDERHILL, Frank P., and Simpson, George Eric. The effect of diet on the excretion of indican and the phenols, 69

Urine and blood, rapid colorimetric methods for the determination of phosphorus, 55

——, estimation of the phenolic substances, 409

——, nature of chlorine combination, 239

VITAMINE, antiberi-beri, a test and its practical application, 487

——, antineuritic, study of the factors which interfere with the use of yeast as a test organism, 113

——, quantitative method in connection with determinations of vitamine in glandular and other tissues, 531

——, water-soluble B, in cabbage and onion, 175

——, —— (B), metabolism studies with diets deficient in, 277

——, ——, in carrots and navy beans, effect of cooking, 159

——, ——, some effects upon nutrition, 255

WATERMAN, HENRY C. See JOHNS and WATERMAN, 303
Water-soluble B in cabbage and onion, 175
—— (B) vitamine, metabolism studies with diets deficient in, 277
—— vitamine in carrots and navy beans, effect of cooking, 159
—— ——, some effects upon nutrition, 255
Wheat plants infected with stem rust, respiration, note, 17
—— ——, respiration of frosted, 13
——, respiration of sprouted, 5
WHIPPLE, BERTHA K. Water-soluble B in cabbage and onion, 175
WISHART, MARY B. Experiments on carbohydrate metabolism and diabetes. III. The permeability of blood corpuscles to sugar, 563

YEAST as a test organism for the antineuritic substance, study of the factors which interfere with the use, 113

ZINC occurrence in marine organisms, 399

NON-CIRCULATING BOOK

RETURN TO→ **CHEMISTRY LIBRARY**
100 Hildebrand Hall 642-3753

LOAN PERIOD 1	2	3
4	5	6

ALL BOOKS MAY BE RECALLED AFTER 7 DAYS
Renewable by telephone

DUE AS STAMPED BELOW

~~AUG 17 1979~~		

UNIVERSITY OF CALIFORNIA, BERKELEY
FORM NO. DD5, 2.5M, 3/78 BERKELEY, CA 94720

U.C. BERKELEY LIBRARIES

C036409352